Development of doubled haploids, chromosome doubling and CRISPR/Cas9 techniques in melon for the next generation of breeding

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«Prêcher la formule de l'eau n'est pas donner à boire à ceux qui ont soif»

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1. GENERAL INTRODUCTION

1.1. CUCURBITACEAE FEATURES and PRODUCTION

1.1.1 Origin and Domestication of cucurbits

The progressive transition of *Homo sapiens* from hunting-gathering to cultivation and animal husbandry began 12,000-10,000 years ago independently in a dozen different geographic regions around the world such as the New World, Africa, the Middle and Near East, Asia and New Guinea (Chomicki et al., 2019; Meyer et al., 2012, and references therein). The development and expansion of agriculture had several triggers such as changes in climate, changes in vegetation, the decrease of prey animal densities, a population expansion, cultural practices and religious beliefs (Harlan, 1992). Agriculture started with the cultivation of wild species as a source of food. Upon their cultivation over the decades, crops progressively diverged from their wild ancestors throughout an evolving process defined as 'domestication syndrome' (Harlan, 1971). Domestication syndrome in plants often involves a set of modified and shared traits such as larger and more sugary fruits, a reduction of physical and chemical defences in the parts used by humans, a change towards a more compact architecture, larger seeds, a reduction in seed dormancy, larger inflorescences and non-shattering seeds (Chomicki et al., 2019; Kantar et al., 2016).

Cucurbitaceae crops were domesticated in several places over the world. The *Cucurbita* genre had a large distribution and adaptation in the pre-Holocene. However, due to the Holocene's megafaunal extinction there was a reduction of wild *Cucurbita*. Archaeological evidence of squash seeds, peduncles and fruit of *Cucurbita pepo* were found in the north of South America and Central America dating from 10,000 years ago (Smith, 1997). An independent domestication occurred in Eastern North America 5,000 years BP (Sanjur et al., 2002; Smith, 2006). From this later domestication, came later all the actual Occidental *C. pepo* cultivars (Pumpkin, Cocozelle, Vegetable Marrow, Zucchini, Scallop, Acorn, Crookneck, and Straightneck Groups). For instance, in the 16th century, *C. pepo* arrived in Italy from America and gave rise to zucchini cultivar in the mid-19th century (Chomicki et al., 2019). *Cucurbita maxima* species was domesticated 4,000 years BP in the coasts of Peru and *C. moschata* in the north of

South America (Sanjur et al., 2002). The earlier archeological evidence of cultivated watermelon seeds were found in Libya dating of 5,000-8,000 years BP (Wasylikowa and van der Veen, 2004). In Asia, *Cucumis sativus* was domesticated from a wild Indian species 2,500 years ago (Qi et al., 2013). *Cucumis melo*, was domesticated two times, first in Egypt 3,700 years ago, and then in China 3,000 years ago (Watson, 1969). The Asian lineage gave rise to the modern cultivars 'Galia', 'Cantaloupe', 'Yellow Honeydew' and 'Piel de Sapo' (Chomicki et al., 2019). In Cucurbitaceae crops, the domestication syndrome included nonbitter fruits, increased fruit size, higher sugar content, higher carotenoid content, decreased physical defences, more compact and less branched growth with increased apical dominance.

1.1.2 Economic importance of cucurbits

Cucurbitaceae are dicotyledon plants belonging to the *Cocurbitales* order. A total of 95 genera and 965 species of *Cucurbitaceae* have been described (Christenhusz and Byng, 2016). From those species, 10 can be classified as 'major crops' due to its global cultivation and economic importance. Another 23 species are considered as 'minor crops' due to its local cultivation and commercial importance (Chomicki et al., 2019). The fruit is the major purpose of *Cucurbitaceae* cultivation which can be consumed raw, ripe, unripe, cooked or pickled. In some species, the seeds and the oil pressed are also valued and used as sweeteners or for their physical and medicinal properties. Most major and minor crops of cucurbit species are herbs with an annual life cycle which may have facilitated domestication, and a monoecious sexual system. However, there are perennial cucurbits too, some of which are cultivated as annuals as a result of management practice. Dioecy appears to be an ancestral state in the family (Chomicki et al., 2019; Zhang et al., 2006).

Despite the huge genetic and morphologic diversity in *Cucurbitaceae*, nowadays, there are three major genres in terms of worldwide production *Citrullus*, *Cucumis* and *Cucurbita,* and few species such as cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), pumpkin and squash (*Cucurbita maxima*), summer squash and zucchini (*Cucurbita pepo*), butternut squash (*Cucurbita moschata*), bottle gourd (*Lagenaria siceraria*) and bitter gourd (*Momordica charantia*) (**FIGURE 1.1**).

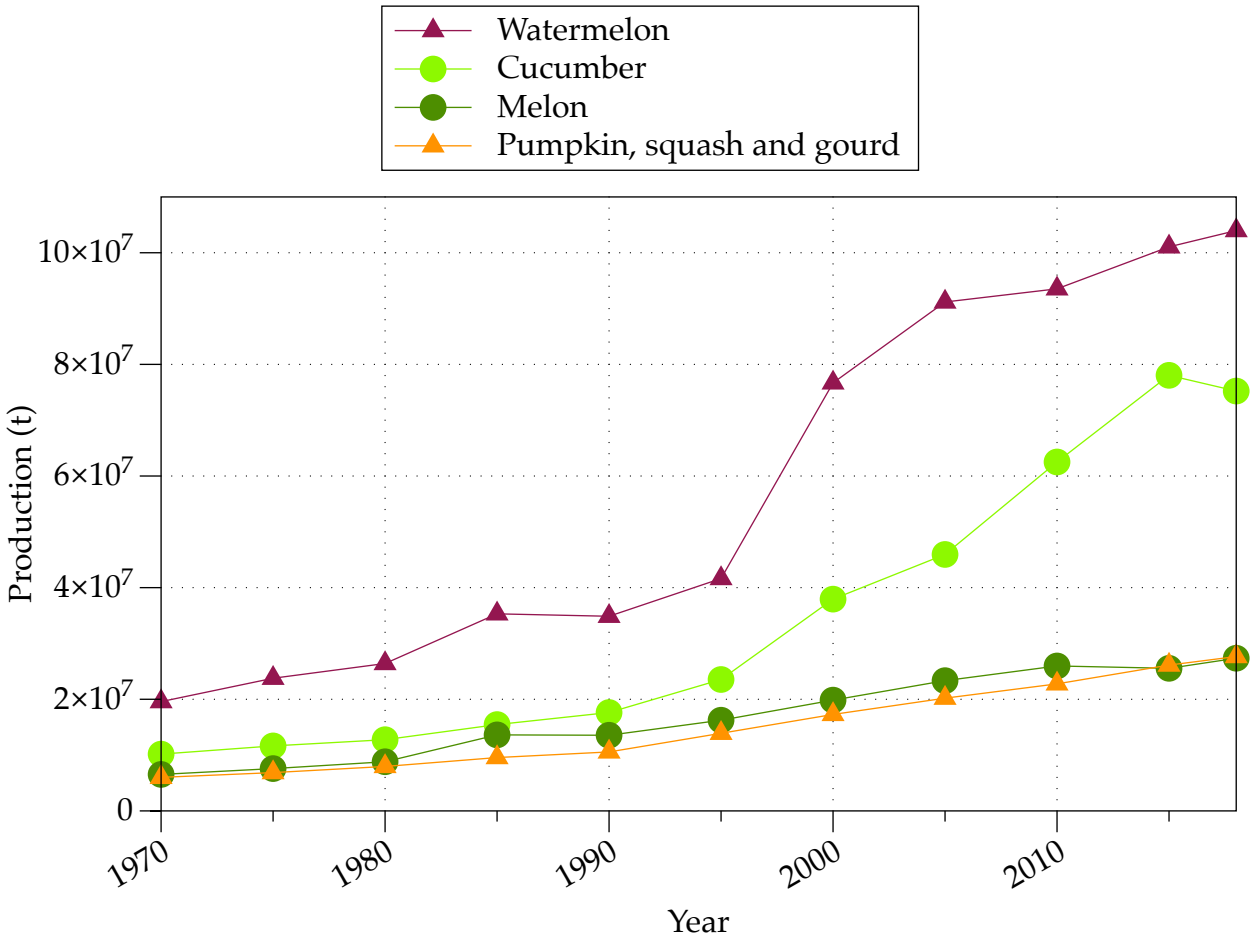

FIGURE 1.1. Worldwide production evolution of Cucurbitaceae species since 'the green revolution' decades (1970) up to nowadays (2018). Dara retrieved from FAO (2018).

In terms of production, watermelon has been the most produced cucurbit crop during the past decades. In 2018, it reached a record production of 103 m tons, followed by cucumber with 75 m tons, melon with 27 m tons, and pumpkins, squash and gourds with 27 m tons (**FIGURE 1.1**). The country that produced the highest amount of cucurbit fruits was Mainland China with 140 m tons. China alone produced the 61.5% of the worldwide production of cucurbit crops. Other countries producers of cucurbits, ordered by its production following China, were India, Iran, Turkey, Russia, US, Mexico, Egypt, Spain and Ukraine. Spain was the 9th world producer in 2018 with a total production of 3 m tons of cucurbit fruits, with 1.092.401 tons of watermelon, 717.645 tons of pumpkins, squash and gourds, 664.353 tons of melon, and 643.661 tons of cucumber (**FIGURE 1.2**).

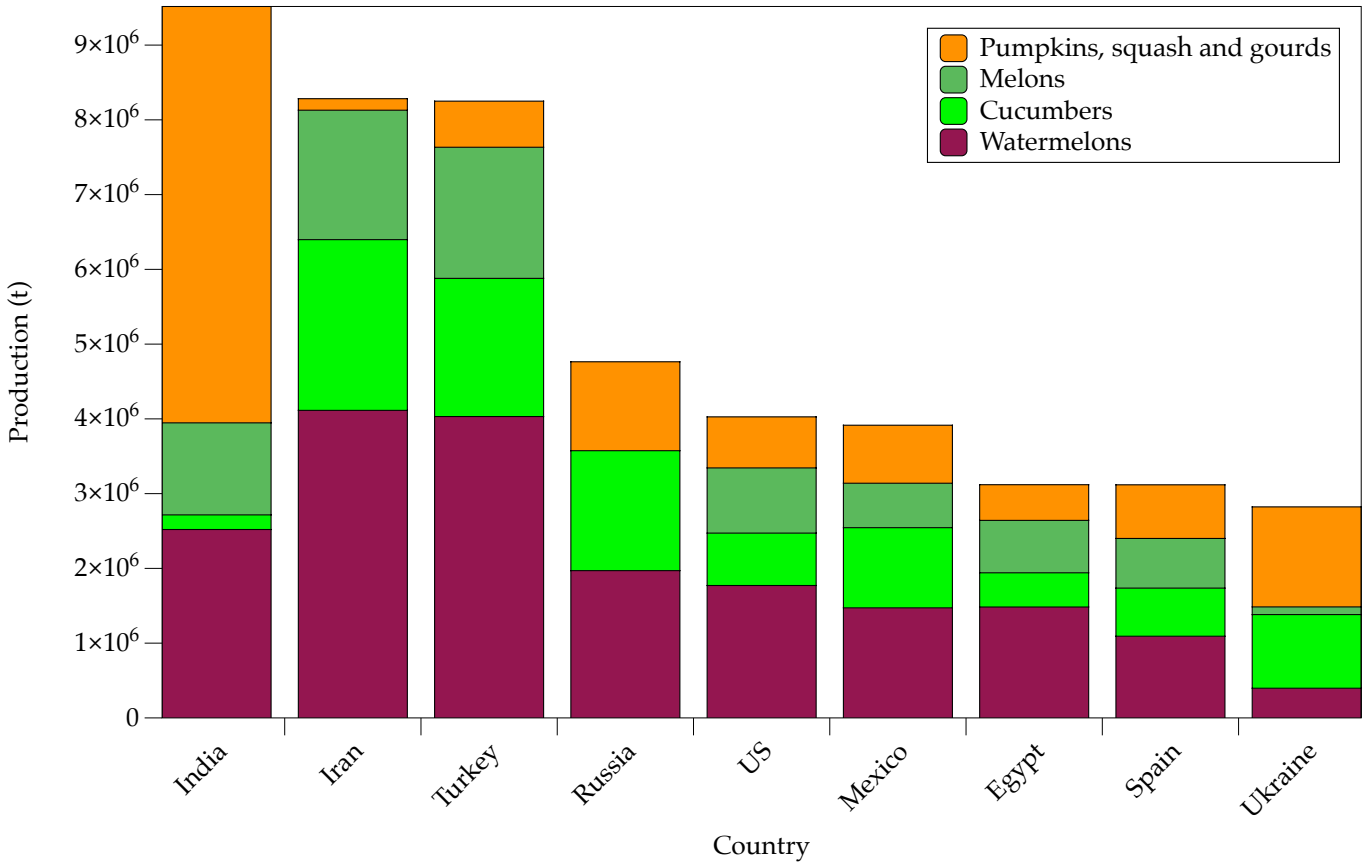

FIGURE 1.2. Most important countries producing Cucurbitaceae species. Excluding Mainland China due to the huge differential production.

*1.2. MELON (*Cucumis melo *L.)*

Melon (*Cucumis melo*) is a diploid species with 2n = 2x = 24 chromosomes. Melon is divided in two subspecies, subsp. *melo* and subsp. *agrestis*. The Occidental melon cultivars such as cantaloupe, galia, honeydew, Western shippers or 'Piel de Sapo' belong to ssp. *melo*. Melon has been divided in 19 groups by Pitrat (2016): *acidulus*, *agrestis*, *ameri*, *cantalupensis*, *chandalak*, *chate*, *chinensis*, *chito*, *conomon*, *cassaba*, *dudaim*, *flexuosus*, *ibericus*, *inodorus*, *indicus*, *kachri*, *makuwa*, *momordica*, and *tibish* (**FIGURE 1.3**).

Melon is cultivated for its fruits and is one of the vegetable crops with a greater fruit diversity. After the initial domestication, melon underwent a huge diversification in different places (Asia and Europe) that led to an extensive diversity of fruit characteristics (Monforte et al., 2014). Size, shape, flesh content, colour, sweetness, aromatic compounds, climacteric behaviour, sourness or taste are examples of the characteristic diversity of melon fruits (**FIGURE 1.3**) (Monforte et al., 2014; Pitrat, 2016).

Nowadays, each country has its own preferences in terms of melon fruit due to cultural reasons (Hooghvorst et al., 2020b). For instance, the preference of melon fruit consumption in Easter Asia (China, Korea, Japan, Mongolia, etc.) is the *makawa* 'Oriental Melon' (**FIGURE 1.3G**); in France is the *cantalupensis* 'European Cantaloupe' (**FIGURE 1.3I**), in Italy is the *inodorus* 'Canary Melon' and the *cantalupensis* 'European Cantaloupe' (**FIGURE 1.3I**); in the US is the *reticultatus* 'North American Cantaloupe' (**FIGURE 1.3J**); in Morocco is the *inodorus* 'Canary Melon; and, in Spain is the *inodorus* 'Piel de sapo' (**FIGURE 1.3A**) and the *cantalupensis* 'European Cantaloupe'(**FIGURE 1.3I**).

FIGURE 1.3. Representative fruits of several melon (*Cucumis melo*) cultivar groups according Pitrat (2008): (**A**) *inodorus* (Piel de Sapo); (**B**) *conomon* (Shiro Uri Okayama); (**C**) *momordica* (PI124112); (**D**) *chate* (Carosello Barese); (**E**) *dudaim* (Queen Anne's pocket melon); (**F**) *acidulous* (TGR-1551); (**G**) *makuwa* (Ginsen Makuwa); (**H**) *ameri* (Kizil Uruk); (**I**) *cantalupensis* (Vedrantais); (**J**) *reticulatus* (Dulce); (**K**) *flexuosus* (Arya); (**L**) *tibish* (Tibish); (**M**) *chinensis* (Songwhan Charmi), and (**N**) wild melon (trigonus). Retrieved from Monforte et al. (2014).

China is the preeminent country in terms of worldwide production of melon, with the 46.54%. It represented a total amount of 13 million tons of melons in 2018 (FAO, 2018). The second most producing melon country was Turkey with 6.5% of the worldwide production. Afterwards, ordered by importance, followed Iran,

India, Kazakhstan, US, Egypt, Spain, Guatemala and Italy. Spain is the 8th producer of melon and produced mostly *inodorus* and *cantaloupensis* melon groups (**FIGURE 1.4**). In 2018, the total production of Spain was 664.353 tons, which represented the 2.53% of the worldwide production. Moreover, its production is very important for the European market, as it supplies the 14% of the European melon demand.

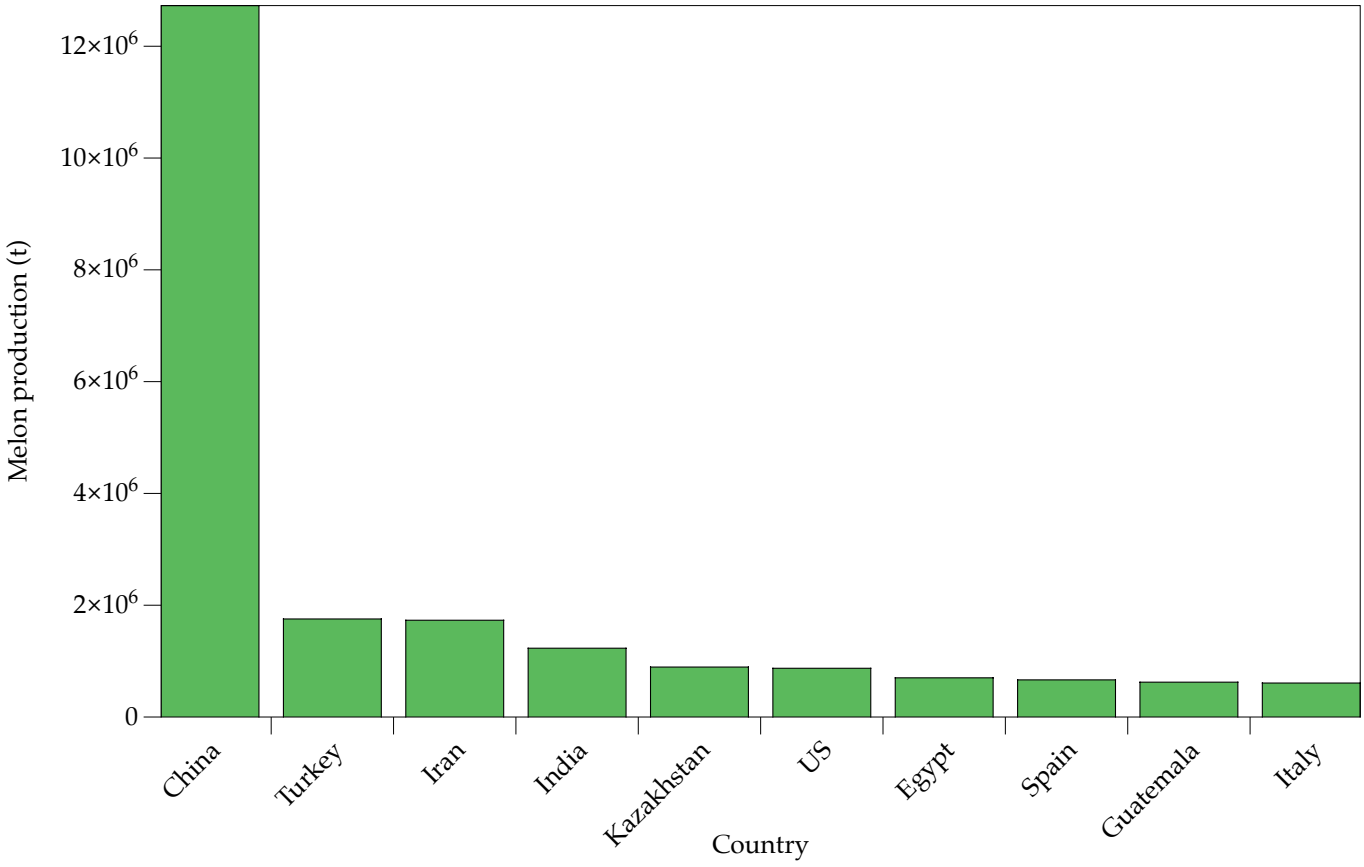

FIGURE 1.4. Most important countries producing melon. Data retrieved from FAO (2018).

1.3. WHAT to BREED FOR?

1.3.1. Yield

Melon production has increased four times since 1970 until nowadays, from 6,535,681 to 27,349,214 tones (FAO, 2018). This incredible rising is due to two main factors: a two-fold increase of the area dedicated to melon production (from 503,194 to 1047,283 ha) and a two-fold increase of yield (from 129,884 to 261,144 hg·ha^{-1}). The experimented boost in yield of the past five decades is a result of several factors such as the extensive use of

hybrids, the improvement of cultural practices, the mechanization of the field, the increased use of chemicals and the use of resistant and highly productive cultivars (**FIGURE 1.5**).

In the coming years, several challenges will have to be addressed in order to keep the production boost emerging from the increasing food supply necessities of an increasing population; together with the challenges that climate change will threat on agriculture.

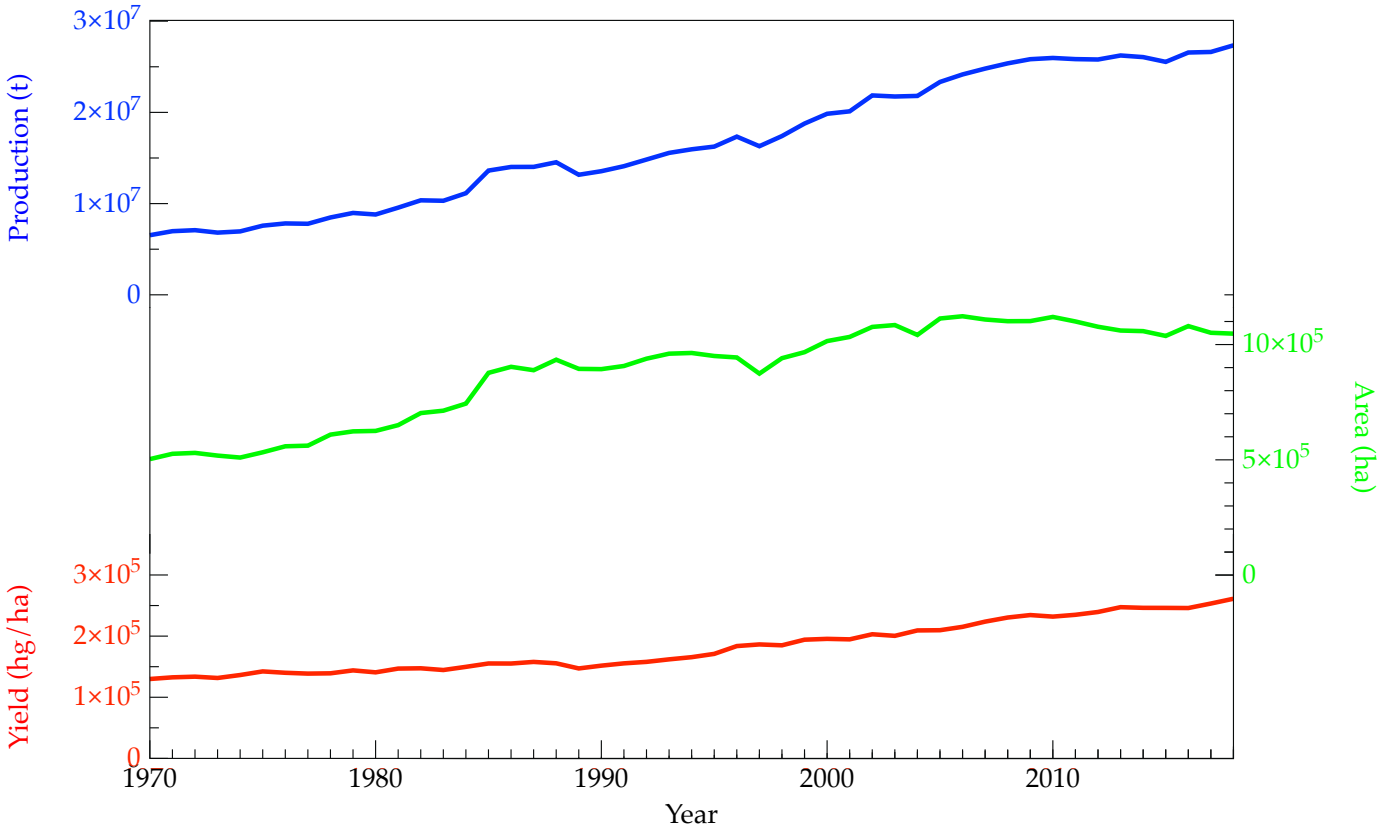

FIGURE 1.5. Changing annual yield (hg·ha^{-1}), land area and production in melon. Both yield and area parameters doubled since 1970, increasing melon production up to 27,349,214 tones. Data retrieved from FAO (2018).

1.3.2. Climate change

Agriculture will suffer a great menace due to climate change during the 21st century. The increasing CO_2 concentration derived from anthropological activity could threaten the current food supply and cause food shortages in the coming years. Different studies claim that climate change will progressively reduce the cultivable

land due to salinization, drought and the rise of sea level. Moreover, together with globalization, the change of climatic regimes in certain areas might increase the chances of novel pests or diseases for crops (Mickelbart et al., 2015; Saadi et al., 2015).

The effects of climate change in the Spanish and European agriculture will be dramatic. Spain represents the 17.83% of the total cultivated area for producing fresh vegetables in the EU28 in 2019 (EUROSTAT, 2019). The cultivation area is highly focused in Almeria, the top vegetable-growing region. This region, together with all the Mediterranean Basin, is predicted to suffer a great desertification and drought process, which will menace the production of one of the most important producers of vegetable and fruit in Europe. The temperature is predicted to increase between 2.10 ºC and 2.80 ºC in the summer and the annual precipitations reduced more than 100 mm annually in 2050 in comparison to 2000 (Saadi et al., 2015). Overall, changes in the crop species and arable area will be mandatory to maintain the actual food production. Species or cultivars with higher tolerance to drought and high temperatures will be cultivated in the more arid regions, and the more demanding species will be moved up to north to more adequate areas (Saadi et al., 2015).

Alongside, an increase of food demand is expected due to the progressive rise of human population predicted to increment from 7.5 billion people in 2018 to 9.7 billion in 2050 (UN, 2019). This scenario coupled with the limitation of food production as a result of climate adversities will be one of the major threats of the coming years.

Climate change is not *per se* a menace because it does not refer to a specific factor; it is a set of predicted variables such as rising temperature, water scarcity, drought, salinity, novel pests or rising sea level, which could directly affect the actual agriculture system. Therefore, breeding crops to cope with climate change effects is a huge task that should focus on numerous traits at once because the effects of climate change will be multivariable.

1.3.3. Diseases and Pathogen Resistances

Melon is grown in all continents except for Antarctica, and the range of pests and diseases affecting it are as widespread as the crop. A huge number of pathogens of different kingdoms such as oomycetes, fungi and its different races, viruses and insects, threaten melon crops year in year out. Over 200 different pathogens have been identified to affect cucurbit species and melon (Zitter et al., 1996). Because pathogenic attacks reduce melon productivity and quality, the final yield can be also reduced between 30 and 50%. Therefore, breeding for pathogen and pest resistance has been one of the major targets in cucurbit breeding.

With the objective to increase resistance in a cultivar, Pitrat (2008) to: (i) assay resistances via artificial inoculation methods, (ii) pyramiding gene resistances to increase the durability of the resistance in front of the variability of the causal pathogen, and (iii) develop cultivars with resistances to several pathogens.

1.3.3.1. Powdery Mildew

Powdery mildew is caused mainly by two fungi *Podosphaera xhantii* and *Golovinomyces cichoracearum* and is one of the major diseases affecting melon. The disease is distinguished by the development of white, powdery mould on both leaf surfaces, on petioles and on stems. The main affecting fungi is *Podosphaera xhantii*, which has several physiological races described. A race is usually based on the phenotypic response to powdery mildew infection shown by a set of differential melon genotypes (McCreight, 2006).

In order to control powdery mildew disease in melon crops there is usually three strategies that can be coupled: (i) chemical treatment for prevention, mitigation and reduction of the fungi, (ii) development of cultural control methods, and (iii) the use of resistant cultivars. Chemical treatments based on strobilurin, myclobutanil, and chlorothalonil fungicides are the most used ones (Keinath and DuBose, 2004). However, the chemical

treatment for controlling the disease has limited success; first, because of the need for new fungicides due to acquired resistance by the pathogen and, second, due to an increasing concern about the drawbacks of using pesticide and chemical treatments. Cultural control such as weed control and good sanitation practices might help reducing the spread and damage of the disease. Nonetheless, the development of resistant cultivars is by far the best approach for powdery mildew control in terms of safety and success.

Normally, resistance genes in melon are dominant and can control several races of *P. xhantii*. However, information about sources of resistance is still confusing and lacks of accuracy due to the minor status of melon as well as several factors affecting the assays such as the genetic variability of the pathogen population, the lack of uniformity in the experimental assays and the significant influence of environmental conditions on the expression of resistance (Cohen et al., 2004). Resistance genes are usually found in accessions from Asia and then pyramided into *Inodorus* or *Cantaloupensis* cultivars. The use of resistant parentals may help the pyramidation of gene resistance into hybrid cultivars thanks to their dominant character.

1.3.3.2. Fusarium wilt

Fusarium wilt disease is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *melonis*. The disease can be characterized by the wilt of the whole plant similar to a water-deficiency symptom (Nogués et al., 2002). Until now, four races have been described: race 0, 1, 2 and 1.2. The resistance to fusarium wilt is usually controlled by few genes which can be easily introgressed into elite cultivars. Those resistance genes are mainly obtained from wild relatives of melon (Oumouloud et al., 2013).

This pathogen can survive in the soil in the form of chlamydospores, which makes the disinfection of contaminated soils very difficult. Nowadays there are mainly four strategies to reduce the impact of fusarium wilt: (i) soil disinfection using chemical products, (ii) soil solarization, (iii) grafting melons into resistant rootstocks, and, (iv) the use of resistant cultivars. The use of chemical products, mainly methyl bromide, has been banned in several developed countries due to health and environmental concerns. The soil solarization method is successful but is not readily applicable for intensive agriculture. The rootstock strategy is very successful for fusarium wilt, but the costs are high and it can only be applied for melon cultivars with high economic value. The use of resistant cultivars is the best method to control fusarium wilt, because they present a complete resistance, however, the costs of producing those cultivars may be high, too.

1.3.3.3. Viruses

There are many viruses affecting melon such as: cucumber mosaic virus, watermelon mosaic virus, zucchini yellow mosaic virus, cucumber vein yellowing virus, cucurbit yellow stunting disorder virus and melon necrotic spot virus. Therefore, they have a great economic impact on melon production. Due to the impossibility to use chemicals to control viruses, the only effective strategy are resistant cultivars. For instance, melon necrotic spot virus (MNSV) is present worldwide, and the only source of resistance is the *nsv* gene, which is actually present in the majority of commercial melon hybrids (Morales et al., 2005).

1.4. BREEDING MELON: METHODS AND TECHNIQUES

Plant breeding is a set of methods and techniques used in order to obtain new cultivars with desired traits beneficial for human or animal consumption. Since the early days of agriculture and the domestication of wild species into crops until nowadays, plant breeding has experienced a great technification. The constant increase of techniques in the breeder's toolbox has enabled to change, improve or delete traits, allowing a great control over plant breeding. Some examples of those techniques are backcross breeding, pedigree breeding, doubled haploids (DHs), molecular breeding, genetically modified organisms (GMO) and genome editing techniques. Some of those methods are in use since the firsts days and some others have been developed during the second half of the 20th century or even in the 21st century. Many are being used for crop improvement, but some new methodologies have been stigmatized and dropped due to social concerns over health issues in certain regions.

The melon breeding has mostly had the attempt to increase the yield, improve the sugar and volatile substances and obtain resistant cultivars to diseases. To reach those objectives many techniques have been applied in melon up to this day. However, with the increasing technification of plant breeding and the emergence of new techniques and approaches, melon and cucurbit species are sometimes lagged in comparison to important crops such as rice, maize and wheat. Nevertheless, in spite of the relative importance of melon, many research and progress has been made.

On the other hand, modern breeding of crops needs homogeneity. The homogeneity paradigm dominates the agriculture and relegates field heterogeneity behind as undesirable since spatial homogeneity allows a precision agriculture. The homogeneity may be accomplished with an inbred line, a DH line or a F1 hybrid. However, this superiority comes at a cost: homogeneous lines aren't diverse populations so they have limited no capacity to undergo long-term adaptation against stresses without the human work (Breseghello and Coelho, 2013).

1.4.1. Classical Breeding

Classical breeding is based on the crossing of close or distant individuals to produce new cultivars with traits of interest. This old but successful way to obtain new cultivars is based on individual plant selection according to the observed variations in comparison to a population. Pedigree selection is the paradigm of classical

breeding which entails a first cross between two stable parents and the following generation of a segregating population which after several generations of selection and self-pollination derive into a set of stable lines with the desired characteristics of both parents. The great majority of cultivars have been developed through the pedigree method (Breseghello and Coelho, 2013).

Backcross breeding is another approach of classical breeding. Based on self-pollination and selection as well, differs from pedigree method on the fact that it attempts to improve an existing cultivar by introgressing a new trait of interest. Normally, the genetic source of this trait of interest comes from another cultivar, a wild relative or an old cultivar. Then, the backcrossing scheme usually starts with the crossing of an elite cultivar and the donor line, with the trait of interest, generating an offspring which is recurrently backcrossed with the elite cultivar at the same time that the trait of interest is selected through generations. By this means, after several rounds of selection and pollination with the elite cultivar, a stable population is obtained with the genetic background of the elite cultivar and the trait of interest of the donor line (**FIGURE 1.**) (Forster et al., 2007).

In spite of its success producing new elite cultivars, classical breeding presents some drawbacks. The need of selecting several generations (between eight and ten) for the trait of interest and the need of controlled pollination make classical breeding very laborious and time-consuming. Besides, to ensure successful results, the number of plants of each population should be considerable high, therefore multiplying the amount of work, installations and volume of plant material. Moreover, the derived line is stable but not completely homozygous. Nowadays, those traditional methods are still applied, usually coupled with other complementary approaches that eases the procedure, such as molecular breeding.

1.4.2. Molecular Breeding

Molecular breeding, or also called marker-assisted selection (MAS), does not represent *per se* a specific methodology of breeding but represents a set of molecular techniques that assist a basic scheme of classical breeding without substituting it. The prerequisites for MAS are DNA markers and a linkage analysis, which identifies a marker linked to a gene controlling a trait. During the process of MAS, the most part of the selection process is trait carried out at an early stage based on a molecular marker linked to a trait. This allows a reduction of the number of individual assessments, dramatically reducing cost and time.

Since the discovery of restriction fragment length polymorphism (RFLP) markers for DNA polymorphism assessment, many others have been developed such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) or single nucleotide polymorphism (SNP). Many of these markers were first discovered for human research and further applied for plant breeding. Molecular markers have been mostly used for gene introgression and gene pyramiding, particularly for disease resistance genes (Ben-Ari and Lavi, 2012).

Although MAS is very successful for breeding monogenic traits, it is not efficient for polygenic traits. Moreover, its use in crop breeding has been restricted to so-called 'major crops' including cereals such as wheat, rice and maize, or vegetables such as tomato and pepper (Ben-Ari and Lavi, 2012; Jannink et al., 2010).

1.4.3. Doubled haploids

DH lines are highly important for plant breeding due to their complete homozygosis, making qualitative and quantitative phenotypic selection more efficient. Since the very first attainment of DHs in *Brassica napus* (Thompson, 1972), many publications have reported the development of DH lines in more than 250 species (Maluszynski, 2003). Following the research conducted in the 70s and 80s that demonstrated the ability to generate DHs in many cereal, vegetable and horticultural crops, the focus in the later decades was to optimize and assay different ways to enhance DH production in each species and genotype by introducing changes and modifications in every step of the DH programs to enhance it.

DH lines are pure and genetically homozygous individuals produced when spontaneous or induced chromosome duplication of haploid cells occurs. DHs are one of the leading achievements in plant breeding because completely homozygous plants can be produced within a year. DH production includes two major steps: haploidization and chromosome doubling. The haploidization attempts to regenerate haploid or spontaneous DH plants, which can be achieved through androgenesis, gynogenesis or parthenogenesis, depending on the species. The chromosome-doubling step is mandatory when spontaneous DHs are not regenerated and is achieved by using antimitotic compounds to double the ploidy level of haploid plants.

Gametic haploid cells are the initial material used to obtain DH lines. Gametes from meiotic cells allow the generation of plantlets when cultured *in vitro* or when pollinated with irradiated pollen. The haploid step can be either a microspore from an anther or an ovule from an ovary depending on the species. The methods for haploidization are androgenesis, gynogenesis or parthenogenesis. Plantlet regeneration from microspores or ovaries is a two-step protocol if a callus step is required prior to plantlet regeneration, or a one-step protocol if it directly induces an embryo or regenerates a plantlet. Besides, gametic cells from meiosis can be developed into haploid embryos, via parthenogenesis. Thus, a DH process always requires a gametic haploid step from which haploid or DH plantlet will be regenerated (**FIGURE 1.**) (Forster and Thomas, 2005).

In vitro culture for gametic cells in androgenesis and gynogenesis techniques allow the original gametophytic pathway of the gamete to be redirected towards a sporophytic pathway where plantlets can be regenerated. On the one hand, androgenesis is the most common method to produce DHs. Isolated microspores or microspores contained in anthers are cultured in specific induction media to induce the formation of callus. Subsequently, these calli are cultured in regeneration media to regenerate fully formed plantlets. In most cereal species, androgenesis is the only or the best method for DH generation with a high rate of spontaneous doubling, such as in rice (Hooghvorst et al., 2018), oat (Kiviharju et al. 2017) and bell pepper (Keleş et al., 2015). On the other hand, gynogenesis stimulates *in vitro* embryogenesis development of the unfertilized haploid egg cells. In this process, a two-step protocol is usually carried out to induce callus formation from the female ovules in induction medium and to regenerate plants from callus in regeneration medium. For onion (Fayos et al. 2015) or beet (Hansen et al. 1995), gynogenesis is the best method for DH production.

The ploidy level of the androgenetic and gynogenetic regenerated plants can differ depending on the cell events related to spontaneous or induced chromosome doubling. Haploids, doubled haploids, mixoploids and tetraploids can be produced during the *in vitro* DH process. In androgenesis and gynogenesis it is desirable that the regenerated plantlets originate from microspore or ovule cells; nonetheless, somatic embryogenesis from anther or ovary tissues can take place. This process is defined as the regeneration of a whole plant from undifferentiated somatic cells in *in vitro* culture. The ploidy of these plantlets is diploid, and the genomic background is identical from which DHs are expected to be generated.

Lastly, parthenogenesis methodology allows the formation of an embryo from an egg cell without fertilization. Egg cells can be induced to develop into haploid embryos following *in situ* pollination with irradiated pollen, and these embryos only inherit the maternal set of chromosomes due to false fertilization. Such embryos germinate *in vitro* and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants. Noteworthy, in the *Cucurbitaceae*, parthenogenesis is the only successful approach to obtain DH plant material (Dong et al., 2016).

All in all, DHs have been of great importance for: establishing chromosome maps and whole genome sequencing in the vast majority of genetically mapped and sequenced species; bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations; and for mapping quantitative trait loci (QTLs) (Forster et al. 2007). This usage of DHs in basic research has been extended to direct application in breeding crops. Furthermore, DHs can be used as commercial cultivars such as stabilized homozygous lines or as parental lines to produce F1 hybrid lines, avoiding classical breeding methods to obtain stabilized and non-segregant lines.

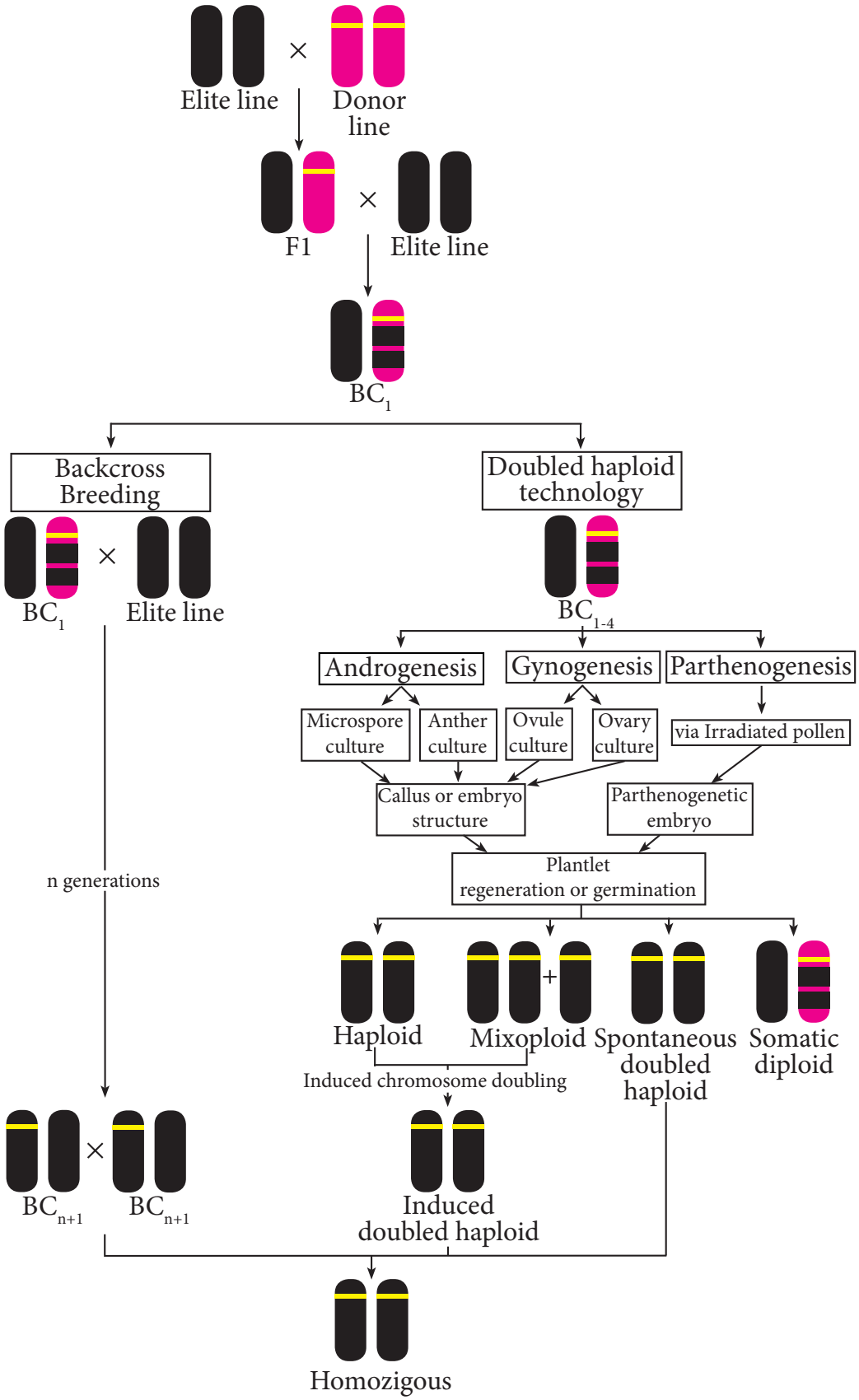

FIGURE 1.6. Schematic representation of backcross breeding and doubled haploid technology. Retrieved and modified from Hooghvorst and Nogués (2020).

1.4.5. Genome editing and CRISPR/Cas9

Genome editing (GE) techniques rely on the genetic engineering of the DNA of a living organism. It is different from genetically modifying (GM) techniques since the first is based in deletions, substitutions or insertions of nucleotides that give the desired feature to the organism, while the second one is based on recombinant DNA for a new feature. There are three main GE techniques: zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats associated to nuclease Cas9 (CRISPR/Cas9). Those techniques rely on an engineered nuclease fused with sequence-specific DNA that can cleave and break a target gene. This damage in the DNA activates the intracellular DNA-repairing mechanisms, which can lead to either non-homologous end-joining (NHEJ) or homologous recombination-based repair (HR).

Nowadays, GE technologies are of major importance in many research areas, plant breeding included. CRISPR/Cas9 technique has risen as the main GE technique thanks to its efficiency and versatility in comparison to previous GE technologies. The complex, time-consuming engineering and unwanted off-target mutations of ZFNs and TALENs have favoured that the CRISPR/Cas9 system to become the genome-editing system of choice. CRISPR/Cas9 has features over its predecessors that make its use and application easier and more efficient (Belhaj et al. 2015).

Since the discovery of CRISPR/Cas9 in 2012, and its first use in plants in 2013 (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013), many applications of GE have been boosted. The CRISPR/Cas9 system enables an unprecedented control over the mutation process thanks to a Cas9 nuclease guided by a 20-nt gRNA sequence (gRNA). The target gene sequence which is complementary to the gRNA sequence must be preceded by a protospacer adjacent motif (PAM). This complex induces DNA double-strand breaks (DSBs) which are repaired by NHEJ generating insertion and deletion events (INDELs) or HR in a precise DNA target sequence.

Importantly, CRISPR/Cas9 based genome editing can be easily applied in crop improvement to enhance yield, nutritional value, disease resistance and other areas. Furthermore, CRISPR/Cas9 can be applied to study and understand the function of genes.

1.5. SEED PRODUCTION

The commercialized melon cultivars can be inbred or F1 hybrids, both of whom present phenotypic stability. Inbred lines are obtained after several rounds of inbreeding through classical breeding and are highly homozygous. The commercial seed of inbred lines is produced by open pollination (OP) with insects, but avoiding the possible cross pollination with other cultivars by culturing in isolated open fields or insect-proof greenhouses. In contrast, F1 hybrids are the heterozygous offspring resulting from the cross of two parental lines which can be inbred or DHs lines. The commercial seed of hybrid F1 lines have to be carefully produced in order to cross the donor line with the receptor line and avoid external pollinations and/or self-pollinations.

1.5.1. OP vs. F1 hybrid

The first studies evaluating the phenotypical advantages of F1 in comparison to their inbred parents were sceptical for the need of extra labour costs of producing the seeds. However, after several studies and assays, melon F1 lines were characterized to have higher yield, higher proportion of flesh, higher soluble solids and earlier production of fruits in comparison to their parental lines (Robinson, 2000). Moreover, the majority of gene resistances are dominant and therefore the production of resistant F1 cultivars can be achieved by using only one resistant parental.

The extra phenotypic performance of F1 is due to heterosis, a phenomenon of the heterozygotic offspring which has enhanced traits as a result of mixing the genetic contributions of its parents. Hybrids usually exhibit greater biomass, speed of development and fertility than both parents. Heterosis phenomenon is greater when the genetic disparity between lines is higher. Several models such as dominance, overdominance and pseudo-dominance have been proposed. Nevertheless, the bases of heterosis are still far from clear and further research needs to be carried out to enlighten this phenomenon (Birchler et al., 2010).

Homozygous melon lines, inbred or DHs, have reduced vigour and yield in comparison to their heterozygous F1 offspring but does not present significant depression of vigour due to inbreeding depression as it does happen in other species. Therefore, the extra labour and cost of F1 seed production is justified because of the higher phenotypical advantages of F1 hybrids and not because of inbreed depression of inbred lines.

1.5.2. Methods for producing F1 hybrid seed

The pollination of melon is naturally performed by insects such as bees, native bees or bumble bees. Likewise, the commercial seed of the inbred cultivars are mostly produced by the pollination of insects in isolated fields or in insect-proof greenhouses, as mentioned above. Nonetheless, the production of the seeds of commercial F1 hybrids need to be looked after. Pollen from the donor parental have to be laid on the female flower of the receptor parental ensuring that the receptor parental does not pollinate itself. The selection of the donor or receptor parental is usually according to the major number of seeds produced. However, to reduce the amount of work and costs that hand pollination represents, gynoecy strategy has been developed.

The phenotypic flowering of each parent is important to produce hybrid F1 seed. Melon is usually andromonoecious but can present others phenotypic flowering pattersn such as gynoecious, monoecious or hermaphrodite. The implications of the phenotypic flowering are important in order to ease the work of pollination or even in terms of the fruit shape as in the monoecious cultivars.

1.5.2.1. Hand emasculation and pollination

Hand pollination is the most widely used method as it does not require technical advances and can be carried out easily. The hermaphroditic flower of the receptor parental is emasculated before anthesis and hand pollinated before dehiscence with the pollen of the male flowers of the donor parental. The pollinated flower should be bagged to avoid insect pollination. This process has several limitations, as its high cost of labour due to the emasculation of receptor's female flowers, collection of donor's male flowers, hand pollination, bagging and carefully avoiding external pollinations is time-consuming and expensive. Moreover, fruit set by hand pollination may be less than by insect pollination. Some culture practices may be coupled to hand emasculation to increase fruit set such as pruning of the main and the secondary apical meristems to promote the development of perfect flowers or the application of growth regulators.

1.5.2.2. Gynoecious parental

The use of a gynoecious parental (only bears female flowers) is an efficient way to produce hybrid seeds. The gynoecious parental as a receptor and the andromonoecious parental as a donor, are crossed simply by

growing them together in a field or a greenhouse with the pollination accomplished by bees. All seed produced should be hybrid, since the gynoecious parental can't self-pollinate due to a lack of pollen. Seedstock of the gynoecious parental are maintained by treating them with growth regulators which induce male flowers development, allowing self-pollination.

2. AIMS & OBJECTIVES

The general aim of this PhD thesis was to study, develop and apply different breeding techniques in melon species in order to produce new commercial cultivars. The three specific objectives of this thesis are:

1. To study and evaluate the commercial value and the parthenogenetic capacity of seven genotypes of *C. melo* var. *Inodorus* "Piel de Sapo" type to obtain DH lines which might be further used as parental lines for commercial hybrid F1 seed production. The parthenogenetic generation of DHs from the seven genotypes was evaluated and optimized through the analysis and description of the different steps of the process, assaying: three haploid embryo rescue protocols, previously described in the literature; three chromosome doubling methods; and, a new cytometry flow method for evaluating the ploidy-level.

2. To develop the applicability of CRISPR/Cas9 system in melon by performing a gene knockout of the melon phytoene desaturase gene (*CmPDS*) in protoplasts and plants.

3. To study and highlight the current methodology in major crops for DH production, the availability of chromosome-doubling methods to obtain DH lines, and the opportunities for HI-mediated genome-editing systems in DH technology. Specifically, the focus was put on haploid inducer-mediated genome-editing systems in cucurbit species to give new insights, opportunities and challenges that may be valuable for developing this technique in cucurbits and other species.

REPORT ON THE IMPACT FACTOR OF THE PUBLISHED ARTICLES

Professor Salvador Nogués Mestres, as supervisor of the thesis entitled "Development of doubled haploids, chromosome doubling and CRISPR/Cas9 techniques in melon for the next generation of breeding" of the PhD student Isidre Hooghvorst, reports on the impact index and participation of the doctoral student in the articles included in his doctoral thesis.

CHAPTER I – "*In situ* parthenogenetic doubled haploid production in melon 'Piel de Sapo' for breeding purposes" by **Isidre Hooghvorst**, Oscar Torrico, Serge Hooghvorst and Salvador Nogués; published in the journal FRONTIERS OF PLANT SCIENCE (4.402 journal impact factor trend 2019).

In this study, the parthenogenetic capacity of seven lines of melon 'Piel de Sapo' type was evaluated in order to produce parental lines of hybrid cultivars. The doctoral student was involved in the study design, experimental and field work, result analysis and the article writing.

CHAPTER II – "Efficient knockout of phytoene desaturase gene using CRISPR/Cas9 in melon" by **Isidre Hooghvorst**, Camilo López-Cristoffanini and Salvador Nogués; published in the journal SCIENTIFIC REPORTS (3.998 journal impact factor trend 2019).

In this study, CRISPR/Cas9 technique was applied, for the first time, in melon targeting *CmPDS* gene producing albino and dwarf plantlets. The doctoral student was involved in the study design, experimental work, result analysis and the article writing.

CHAPTER III – "Chromosome Doubling Methods in Doubled Haploid and Haploid Inducer-Mediated Genome-Editing Systems in Major Crops" by **Isidre Hooghvorst** and Salvador Nogués; published in the journal PLANT CELL REPORTS (3.825 journal impact factor trend 2019).

In this review, the DH methodologies, the availability of chromosome-doubling methods to obtain DH lines, and the opportunities for HI-mediated genome-editing systems in DH technology are summarized for major crops. The doctoral student proposed the idea and wrote the manuscript.

CHAPTER IV – "Opportunities and Challenges in Doubled Haploids and Haploid Inducer-Mediated Genome-Editing Systems in Cucurbits" by **Isidre Hooghvorst** and Salvador Nogués; published in the journal AGRONOMY (1.683 journal impact factor trend 2019).

In this review, the DH methodologies, the availability of chromosome-doubling methods to obtain DH lines, the CRISPR/Cas9 experiments, and the opportunities for HI-mediated genome-editing systems in DH technology are summarized for cucurbit species. The doctoral student proposed the idea and wrote the manuscript.

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Prof. Salvador Nogués

Thesis supervisor

3. RESULTS

CHAPTER I. *In situ* parthenogenetic doubled haploid production in melon 'Piel de Sapo' for breeding purposes

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ABSTRACT

Doubled haploids in cucurbit species are produced through *in situ* parthenogenesis via pollination with irradiated pollen for further use as parental lines for hybrid F1 production. In this study, seven genotypes of melon "Piel de Sapo" were appraised for agronomic traits and pathogen resistances to evaluate its commercial value and used as donor plant material for the parthenogenetic process. Then, *in situ* parthenogenetic capacity of melon "Piel de Sapo" germplasm was evaluated and optimized. Several steps of the parthenogenetic process were assessed in this study such as melon fruit set after pollination with irradiated pollen, haploid embryo obtention, *in vitro* germination and growth of parthenogenetic embryos and plantlets, *in vitro* and *in vivo* chromosome doubling with colchicine or oryzalin and fruit set of doubled haploid lines. Parthenogenetic efficiencies of "Piel de Sapo" genotypes showed a high genotypic dependency during the whole process. Three different methods were assayed for parthenogenetic embryo detection: one-by-one, X-ray and liquid medium. X-ray radiography of seeds was four times faster than one-by-one method and jeopardized eight times less parthenogenetic embryo obtention than liquid medium. One third of melon fruits set after pollination with irradiated pollen contained at least one parthenogenetic embryo. The 50.94% of the embryos rescued did not develop into plantlets because failed to germinate or plantlet died at the first stages of development because of deleterious gene combination in haploid homozygosity. The distribution of the ploidy-level of the 26 parthenogenetic plantlets obtained was: 73.08% haploid, 23.08% spontaneous doubled haploid and 3.84% mixoploid. Two *in vitro* chromosome doubling methods, with colchicine or oryzalin, were compared with a third *in vivo* colchicine method. *In vivo* immersion of apical meristems in a colchicine solution for 2 h showed the highest results of plant survival, 57.33%, and chromosome doubling, 9.30% mixoploids and 20.93% doubled haploids. Fruit set and seed recovery of doubled haploids lines was achieved. In this study, doubled haploid lines were produced from seven donor genotypes of melon "Piel de Sapo," however, further improvements are need in order to increase the parthenogenetic efficiency.

INTRODUCTION

Melon (*Cucumis melo*) is a eudicot diploid plant species from *Cucurbitaceae*. Melon has been divided in two subspecies, subsp. *melo* and subsp. *agrestis*, and 19 groups have been described by Pitrat (2016): *acidulus*, *agrestis*, *ameri*, *cantalupensis*, *chandalak*, *chate*, *chinensis*, *chito*, *conomon*, *cassaba*, *dudaim*, *flexuosus*, *ibericus*, *inodorus*, *indicus*, *kachri*, *makuwa*, *momordica* and *tibish*. Together with cucumber (*Cucumis sativa*) and watermelon (*Citrullus lanatus*), melon is one of the most economic important species from Cucurbitaceae. Melon production was about 32 million tons in 2017 (FAO, 2017), being China, Turkey, Iran, Egypt, India, Kazakhstan, USA and Spain, the major producers ordered according to its yield. The melon fruit has a huge genotypic diversity, and each country has its own preferences due to cultural reasons (Monforte et al., 2014). *Inodorus* and *Cantalupensis* are the most produced melon groups in Spain. Pathogens are a major threat to melon productivity, Zitter et al. (1996) estimated that over 200 pathogens affected the productivity of cucurbits, caused by fungi, bacteria, viruses or mycoplasma organisms. It is estimated that diseases can cause yield losses of more than 30-50% in melon cultivation (El-Naggar et al., 2012). Powdery mildew, fusarium wilt and melon necrotic spot virus (MNSV) are the most critical diseases in melon and cucurbit species. Consequently, to the high impact of pathogens in cucurbits many modern breeding programs have been implemented to obtain resistant cultivars (Kuzuya et al., 2003; Lotfi et al., 2003).

Commercial seed of melon cultivars can be open pollination cultivar (OP) or hybrid F1 (Robinson, 2000). OP cultivars are inbred lines obtained through several rounds of self-crossing until the obtention of a high homozygous and stable line. On the other hand, hybrid F1 cultivars are stable but heterozygous lines obtained from the cross of two homozygous lines. Hybrids F1 take advantage of heterosis for major fruit yield and pathogen resistances and have a great importance in the European market in spite of its production costs (McCreight et al., 1993; Robinson, 2000). Hybrids F1 are produced by crossing two pure parental lines, which can be obtained by successive rounds of self-crossing and selection during classical breeding or by biotechnology approaches, like doubled haploids (Dong et al., 2016). Doubled haploids (DHs) are pure homozygous lines which require shorter time to produce in comparison to classical breeding (Germanà, 2011). DH lines are generated by androgenesis, gynogenesis or parthenogenesis in major crops, and can be used as a parental for hybrid F1 production or as a stable line. In cucurbit species, *in situ* parthenogenesis through irradiated pollen is the most common and efficient

method to obtain haploid plants (Sauton and Dumas de Vaulx, 1987). Those haploid plants need to undergo chromosome doubling by antimitotic compounds. *In situ* parthenogenesis in cucurbits to produce haploid embryos is usually low, from 0 to 5% of seeds contain haploid embryos (Dong et al., 2016), and is less efficient and more time-consuming than other crop species such as: wheat (Niu et al., 2014), bell pepper (Irikova et al., 2011), rice (Hooghvorst et al., 2018) or onion (Fayos et al., 2015).

First haploids of melon, embryos and plants, were obtained using an interspecific crossing with *Cucumis ficifolius* (Dumas de Vaulx, 1979). Then, *in situ* production of haploid embryos was achieved thought pollination with irradiated pollen (Sauton and Dumas de Vaulx, 1987). The pollination of a female flower stigma with irradiated pollen stimulates an *in situ* parthenogenetic response when pollen tube reaches the egg-cell. Then, parthenogenetic haploid embryo is developed, extracted and cultured *in vitro*. Germinated embryo regenerates into a full-developed plantlet that need to undergo chromosome duplication for DH seed recovery. Nevertheless, *in situ* parthenogenesis in cucurbits and specifically, in melon, has many bottlenecks that reduces its efficiency in each step of the process. Melon parthenogenesis has a high genotypic dependency and methodological issues that impede the efficient production of DHs such as: low levels of female flowers developed once pollinated with irradiated pollen; low production of haploid embryos; difficulty to detect seeds containing haploid embryos; low germination of haploid embryos *in vitro*; high mortality of germinated embryos and growing plantlets; very low or null spontaneous chromosome duplication; difficulty to induce chromosome doubling in haploid plants due to a high mortality and hyperhidricity; high ratio of haploid and mixoploid plants; low pollen germination levels of chromosome doubled plants which trigger a decrease of fruit set and seed recovery; and, low DH seed germination (Dong et al., 2016; Gonzalo et al., 2011; Lim and Earle, 2008, 2009).

The seeds of melon fruits set produced via pollination with irradiated pollen are inspected in search of parthenogenetic embryos. The inspection of seeds one-by-one under a stereo microscope is successful and the most commonly applied although its time-consuming and labor-intensive. Two other methods have been reported for parthenogenetic haploid embryo detection such as X-rays, which had been proven efficient but demand high equipment specialization, and liquid culture, which had been proven ineffective (Dong et al., 2016). The low rate of spontaneous chromosome doubling during melon parthenogenesis process require the implementation of a chromosome doubling step using antimitotic compounds. Colchicine has been the most used antimitotic in melon

for chromosome doubling, either via immersion of *in vitro* shoot tips or nodular explants, or via immersion of *in vivo* shoot tips. Chromosome doubling rate can range from 0% to 90% depending on the genotype (Gonzalo et al., 2011; Lim and Earle, 2008; Solmaz et al., 2011; Yetisir and Sari, 2003).

The main objective of this study was to evaluate the commercial value and the parthenogenetic capacity of seven genotypes of *Cucumis melo* var. *Inodorus* "Piel de Sapo" type to obtain DH lines which will be further used as parental lines for commercial hybrid F1 seed production. Moreover, the parthenogenetic generation of DHs from the seven genotypes was evaluated and optimized through the analysis and description of the different steps of the process, assaying: three haploid embryo rescue protocols, previously described in the literature; three chromosome doubling methods; and, a new cytometry flow method for evaluating the ploidy-level.

MATERIALS AND METHODS

Plant material and growth conditions

Seven genotypes of *Cucumis melo* subsp. *melo* 'Piel de Sapo' *indodorus* type were used as plant material (provided by ROCALBA S.A.). Six genotypes were inbred lines (PS-1305, PS-1901, PS-0301, PS-0709, PS-2001, and PS-2301) and one genotype was an open pollinated cultivar (Melito). Melon plants were grown in greenhouse conditions at Servei de Camps Experimentals at the *Universitat de Barcelona* (Barcelona, Spain) in 9 L plastic containers filled with substrate containing Floratorf peat moss (Floragard Vertriebs, Oldenburg)-vermiculite (2:1 v/v) substrate supplemented with Osmocote (The Scotts Company LLC, USA) and one gram $CaCO_3$ per peat liter was added to adjust the substrate pH to 6 (**FIGURE 3.1.1.A**).

Pollination with irradiated pollen, parthenogenetic embryo rescue and germination

Male flowers containing mature pollen were collected early in the morning and irradiated at 250 Gy using a ^{137}Cs source at *Centres Científics i Tecnològics* at the *Universitat de Barcelona* (Barcelona, Spain). Female flowers were emasculated, pollinated with the help of a brush, and bagged to avoid external pollinations. Each female flower receptor was pollinated with three to five irradiated male flowers (**FIGURE 3.1.1.B**). Pollination was done at the same and the next day of irradiation. After three weeks, melon fruits that set were harvested and opened for seed collection. Three different methods of seed inspection and embryo detection and rescue were assayed: seed inspection one-by-one with the help of a light box, X-ray radiography of seeds and floating seeds in liquid medium. In the one-by-one individual inspection of seeds method, seeds were sterilized in 20% sodium hypochlorite supplemented with 4 drops·L^{-1} of Tween 20, rinsed three times in sterile water and opened one-by-one with the help of a stereo binocular microscope and a light box. In the detection of embryos by X-ray radiography method, seeds were placed in an acetate sheet (**FIGURE 3.1.1.E**) on the Imaging Screen K, introduced in a Faxitron® cabinet X-Ray system (Hewlett Packard, California, USA) to be exposed to 16 kV during 70 s and the seeds containing embryo were selected and sterilized (**FIGURE 3.1.1.F**). For liquid culture of seeds, seeds were sterilized and cultured *in vitro* in E20A liquid medium in jars. Detected seeds containing embryos with the three

methods were manually opened in aseptically conditions and embryos were cultured *in vitro* in E20A solid medium (Sauton and Dumas de Vaulx, 1987) in 90 mm petri dishes.

Chromosome doubling

Three different protocols were assayed to induce chromosome doubling in haploid plantlets, two *in vitro* and one *in vivo*. The two *in vitro* treatments used nodes and shoot tips as plant material, the protocol of Lim and Earle, (2009) applied 500 $mg \cdot L^{-1}$ of colchicine for 12 h; and, the protocol of Ebrahimzadeh et al. (2018) applied 50 $mg \cdot L^{-1}$ of oryzalin for 18 h. Each chromosome doubling method was performed three to five times in different days to treat them as independent replicates. For both *in vitro* treatments, *in vitro* haploid plantlets were micropropagated aseptically, nodes with two to three axillary buds and shoot tips with one to two axillary buds were treated in E20A liquid medium supplemented with the antimitotic solution and DMSO 2% (v/v) during the correspondent exposition time. Once the exposition time elapsed, nodes and shoot tips were rinsed with water and cultured in E20A solid medium. The third chromosome doubling protocol assayed was *in vivo*, following the methodology of Solmaz et al. (2011) and Yetisir and Sari (2003). *In vitro* haploid plantlets were acclimated in the greenhouse (**FIGURE 3.1.1.J**) and when plants expanded four to eight leaves, apical stem was submerged in a 5000 $mg \cdot L^{-1}$ colchicine solution supplemented with 2 $drops \cdot L^{-1}$ of Tween 20 for 2 h (**FIGURE 3.1.1.K**). Apical stems were rinsed with water after the treatment.

When apical stems expanded new leaves since the application of the antimitotic treatment, ploidy-level was determined to identify the induced chromosome doubled plants. *In vitro* chromosome doubled plantlets that survived and grew roots were acclimatized in a growth chamber at 25 °C, illuminated with 50-70 $\mu mol\ m^{-2}\ s^{-1}$ fluorescent light under a 16/8 h day/night photoperiod and covered with plastic pots. After two weeks, the plantlets that survived were acclimatized in the greenhouse (**FIGURE 3.1.1.J**). The stems of *in vivo* treated plants that remained haploid were pruned, and the ones that chromosome doubled were grown until flowering and self-pollinated. If plants showed phenotypical mixoploidy, carrying male flowers with and without pollen, stems with haploid flowers were pruned.

Ploidy-level determination

The ploidy-level of germinated parthenogenetic embryos and antimitotic treated plants was determined by flow cytometry following the procedure of Hooghvorst et al. (2018) with slight modifications. About 5 mg of young leaves were collected and put into ice-cold 2 mL microcentrifuge tubes each with a steel bead (3 mm diameter). To each tube, 300 µL of cold lysis buffer (0.1 M citric acid and 0.5% Triton X-100 in distilled water) were added. Tubes were cooled at -20ºC for 10 min. Samples were shaken at 25 Hz for a total of 20 s in a MM 400 tissue lyser (Retsch, Haan, Germany) two times and tubes were vortexed with a vortex mixer between the two trituration. The aliquot from each tube was filtered through a 22 µm nylon filter (Sefar Maissa, Blacktown, Australia), gently vacuumed and transferred to a flow cytometry sample tube (Beckman Coulter Inc., Pasadena, California, USA). Afterwards, 150 µL of propidium iodide (PI) stain solution [0.25mM Na_2HPO_4, 10 mL 10x stock (100 mM sodium citrate, 250 mM sodium sulphate) and 9 M PI made up to 100 mL with Milli-Q water] was added to each tube. Tubes were then sealed and kept on ice in the dark for 1 h before flow cytometry analysis. The stained nuclei samples were analysed using a GalliosTM Flow Cytometer (Beckman Coulter Inc., Pasadena, California, USA) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centers, University of Barcelona) and a 32-well carrousel. One diploid control sample was included every seven measurements. Flow cytometry data was analysed using Summit Software v4.3 (Beckman Coulter Inc., Pasadena, California, USA).

DH haploid seed recovery

The acclimated and chromosome doubled plants were grown in the greenhouse. When melon plants flowered, self-pollination of DH plants was done by pollinating the emasculated female flowers with three to five male flowers. Once female flowers were pollinated, they were bagged and three days later the viability was checked. After five weeks since pollination melon fruits set were collected, and DH seed was recovered.

Evaluation of melon fruit traits

Melon fruit traits of donor genotypes were evaluated at commercial maturity stage. The evaluated traits were: fruit weight (FW); fruit diameter (FD); fruit length (FL); fruit shape (FL/FD); fruit skin netting (FSN),

evaluated as 0 (no netting), 1 (low netting), 2 (moderate netting), 3 (high netting) and 4 (very high netting); sugar content (ºBrix); and, fruit aroma, evaluated as 0 (no aroma), 1 (good aroma), 2 (very good aroma) and 3 (excellent aroma). The fruit aroma evaluation was done by a board of experts. Five to eight melon fruits were evaluated in each genotype.

Powdery mildew, Fusarium wilt and MNSV evaluation

The resistance or susceptibility of the seven donor genotypes was evaluated for *Podosphaera xhantii*, *Fusarium oxysporum* f.sp. *melonis*, and, melon necrotic spot virus (MNSV). Five races of powdery mildew fungi *Podosphaera xhantii* (Px) were evaluated 1, 2, 3, 3.5 and 5. Fungi material was provided by GEVES (France). Plants were grown in the greenhouse until expansion of the third true leaf. Leaf disks of 9 cm diameters were taken from the first true leaf, disinfected in 20% sodium hypochlorite supplemented with 4 drops·L^{-1} of Tween 20 for 20 s, rinsed twice in sterile distilled water and plated into 90 mm petri dishes filled with powdery mildew medium, 25 mg·L^{-1} of Benzimidazole and 1.6 g·L^{-1} agar. Each leaf disk was inoculated manually with five conidiophores and up to ten leaf disks were analysed in each genotype. After 12-15 days upon infection, each leaf disk was phenotypically evaluated according to the sporulation level using a scale from 0 to 4: 0, no sporulation; 1, hyphae present without no conidiophores and yellowing leaf disk; 2, hyphae present and up to twenty conidiophores; 3, 20 to 100 conidiophores present; 4, more than 100 conidiophores. Level 0 and 1 were considered as resistant, and levels 2 to 4 were considered as susceptible.

Resistance or susceptibility to *Fusarium oxysporum* f.sp. *melonis* (*Fom*) fungi races 0, 1 and 2 was evaluated. The fungi material was provided by BCCM (Belgium). Plants were grown in the greenhouse until third true leaf was expanded. Then, plants were removed from the substrate, the roots were washed with tap water and soaked for 30 s in a fungi solution of $3 \cdot 10^6$ spores·mL^{-1}. After the infection, plants were planted again in the substrate. Five plants were analysed in each genotype. After 30 days upon infection, plants were evaluated according to fusarium wilt symptoms using a scale from 0 to 4: 0, plant without disease; 1, low levels of wilting and yellowing leaves; 2, leaves heavily affected by wilting; 3, all leaves wilted; 4, dead plant. Level 0 and 1 were considered as resistant, and levels 2 to 4 were considered as susceptible.

For melon necrotic spot virus (MNSV) evaluation, plants were grown in the greenhouse until the expansion of the third true leaf. Then, virus inoculation was carried out by mechanical inoculation on the cotyledon with a solution of 0.03M Na_2HPO_4, 0.2% DIECA, 75 $g \cdot L^{-1}$ carborundum and 1 g of leaf infected with MNSV. Once the inoculation was made, plants were grown at 22ºC and 50-70 $\mu mol\ m^{-2} \cdot s^{-1}$ fluorescent light under a 16/8 h day/night photoperiod. Five plants were analysed in each genotype. After 20 days upon infection, plants were evaluated according to the presence of virus symptoms using a scale from 0 to 3: 0, no symptoms present; 1, presence of few necrotic spots in the cotyledons; 2, presence of necrotic spots in cotyledons and true leaves, and malformation of new expanded leaves; 4, collapsed plant.

Statistical Analysis

The parameters FW, FD and FL were analysed using ANOVA One-Way test with a post-hoc Bonferroni test ($P<0.05$). The Brix (º) parameter was analysed with Kruskal-Wallis with a post-hoc Dunn's test ($P<0.05$). The parameters FSN and fruit aroma and the parthenogenesis efficiencies were analysed with Chi Square test ($P<0.05$). All experiments were established in a completely randomized design.

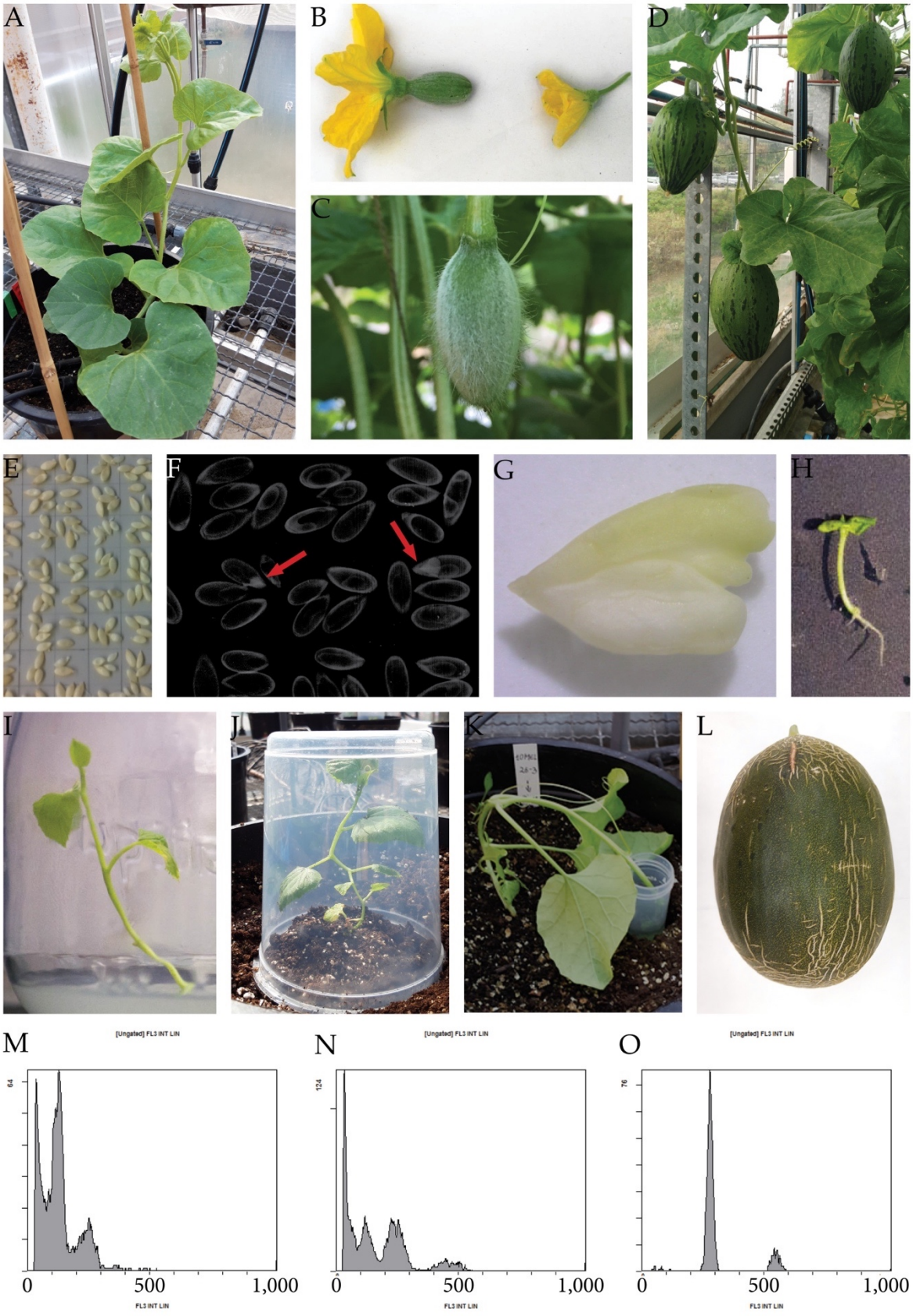

FIGURE 3.1.1. Production of parthenogenetic DH lines in melon "Piel de Sapo" genotype. (**A**) Melon donor plant. (**B**) Detail of a female flower (left) and a male flower (right). (**C**) Female flower developed after pollination with irradiated pollen. (**D**) Melon fruits set after three weeks of pollination with irradiated pollen, which potentially contain parthenogenetic embryos in their seeds. (**E**) Melon

seeds in an acetate sheet ready to be photographed by X-ray. (**F**) X-ray photography of seeds, parthenogenetic embryos are pointed with a red arrow. (**G**) Parthenogenetic embryo rescued. (**H**) Germinated embryo *in vitro*. (**I**) Micropropagated melon plantlet. (**J**) Acclimated melon plant covered with a plastic pot to avoid dryness. (**K**) *In vivo* chromosome doubling treatment of a haploid plant by immersion of the apical meristem into a colchicine solution. (**L**) Melon fruit of a DH line. (M) Flow cytometry histogram of a haploid plant, showing ploidy-peaks at channels 150 and 300, corresponding to haploid cells in G0 phase and G2 phase, respectively. (**N**) Flow cytometry histogram of a mixoploid plant, showing ploidy-peaks at channels 150, 300, and 600, corresponding to haploid cells in G0 phase, diploid cells in G0 phase and haploid cells in G2 phase, and, diploid cells in G2 phase, respectively. (**O**) Flow cytometry histogram of a DH plant, showing ploidy-peaks at channels 300 and 600, corresponding to diploid cells in G0 phase and G2 phase, respectively.

Evaluation of parental donor genotypes

Agronomic traits of the seven donor genotypes were evaluated in a random designed experiment during two consecutive years, 2017 and 2018 in test fields property of ROCALBA S.A. located in Monzón, Spain (**TABLE 3.1.1**). The fruit length (FL) of the evaluated genotypes ranged around 23 cm excepting Melito genotype which had a statistically different length ($P<0.05$) of 19.82±1.35 cm. Fruit diameter (FD) showed significant differences between genotypes ($P<0.05$), Melito had the smallest diameter and PS-0709 the widest. The shape parameter correlates FL and FD and therefore, express if fruits are ovate, elliptic or elongated when higher or lower the ratio. The PS-0301 genotype showed the most elliptical melon fruits. The fruit weight (FW) of melon fruits was relatively stable inside the same genotype. Nevertheless, significant differences ($P<0.05$) between genotypes were found, Melito showed the lightest melon fruits (1.83±0.35 kg) and PS-0809 the heaviest melon fruits (3.81±0.77 kg). Sugar content, measured in Brix (º), and aroma are independent, higher values of sugar do not entail a better flavor. Melito and PS-1305 were considered as the best genotypes in terms of flavor because of the high values of aroma and sugar. No significant differences ($P>0.05$) were found for the Kruskal-Wallis test analysing FST and aroma due to a low number of replicates and the reduced range of the parameter. Pathogen resistance evaluation showed a high number of resistances in most of the genotypes excepting Melito, which was only resistant to *Fom* race 2 and sensitive to the other pathogens and races assayed. The genotypes PS-1305, PS-0301, PS-0709, PS-2001 and PS-2301 showed resistances for all *Px* and *Fom* races evaluated. However, resistance to MNSV was only present in PS-1305, PS-0709 and PS-2301.

TABLE 3.1.1. Fruit trait evaluation and pathogen resistance analysis of the seven genotypes of melon 'Piel de Sapo'.

	PS-1305	**PS-1901**	**PS-0301**	**PS-0709**	**PS-2001**	**Melito**	**PS-2301**
FL	23.53 ± 20^{a}	23.26 ± 1.86^{a}	24.88 ± 1.70^{a}	23.16 ± 1.94^{a}	22.81 ± 3.46^{a}	19.82 ± 1.35^{b}	23.82 ± 1.72^{a}
FD	14.28 ± 1.42^{abc}	15.63 ± 1.86^{abc}	15 ± 1.65^{abc}	17.37 ± 1.82^{d}	15.47 ± 2.13^{abc}	13.11 ± 1.35^{ac}	14.45 ± 1.71^{abc}
Shape	1.65	1.49	1.66	1.33	1.47	1.51	1.65
FW	2.58 ± 0.36^{a}	3.26 ± 0.69^{ab}	2.86 ± 0.50^{a}	3.81 ± 0.77^{bc}	3.29 ± 1.13^{ac}	1.83 ± 0.35^{d}	3.07 ± 0. 59^{a}
FSN	1	2	2	2	1	0	1
Aroma	3	3	1	1	2	2	2
Brix (º)	14.17^{ab}	13.92^{abd}	12.31^{c}	13.47^{bd}	12.69^{cd}	14.47^{abd}	13.27^{bcd}
***Fom* 0**	R	S	R	R	R	S	R
***Fom* 1**	R	R	R	R	R	S	R
***Fom* 2**	R	R	R	R	R	R	R
MSNV	R	S	S	R	S	S	R
***Px* 1**	R	R	R	R	R	S	R
***Px* 2**	R	R	R	R	R	S	R
***Px* 3**	R	S	R	R	R	S	R
***Px* 3-5**	R	R	R	R	R	S	R
***Px* 5**	R	R	R	R	R	S	R

Parameters followed by * presented significantly differences (P<0.05) and values followed same letter showed no significance (P>0.05).

Pollination with irradiated pollen and parthenogenetic embryo rescue

Seven to eight plants of each genotype were grown in the greenhouse to be used as donor plant material (**FIGURE 3.1.1.A**). A total of 1,128 flowers were pollinated with irradiated pollen and 178 of them developed melon fruit (**FIGURE 3.1.1.C,D**). After pollination, some flowers initially developed but later failed to fruit set and finally aborted. A previous experiment had been carried out to analyse the germination of the irradiated pollen and the ability to fruit set. The irradiated pollen germinated correctly and set melon fruit when the female flower was pollinated the same and the next day upon irradiation. More days of storage or different storages reduced dramatically the germination of pollen and the fruit set (data not shown). Analysing the seven genotypes, significant differences ($P<0.05$) were found for melon fruit set between genotypes using a Chi Square test. The PS-0301 genotype had the highest fruit set (24.6%) and PS-0709 the lowest (9.7%) (**TABLE 3.1.1**). Melon fruits of three weeks old since pollination were collected and opened for parthenogenetic embryo rescue. Three different protocols were assayed to seek parthenogenetic embryos: one-by-one, X-ray radiography and floating seeds in liquid medium (**TABLE 3.1.3**). The seeds of 28 melon fruits were opened using the one-by-one method and eight parthenogenetic embryos were found. On the other hand, 127 melons were opened by X-ray radiography and 44 parthenogenetic embryos were found. Finally, 23 melons were opened by floating seeds in liquid medium and one parthenogenetic embryo was found. The percentage of detected melons carrying parthenogenetic embryos was similar between one-by-one and X-ray methods, 28.57% and 34.65%, respectively. Nevertheless, X-ray method was found to be four to five-times faster than one-by-one method due to seeds containing parthenogenetic embryo were the only ones opened (**FIGURE 3.1.1.E,F**). When floating seeds in liquid medium, only 4.35% of melon fruits were contained parthenogenetic embryos due to many of the cultured seeds in liquid medium were contaminated and therefore discarded, despite the initial decontamination of seeds with bleach. Overall, a total of 53 parthenogenetic embryos (**FIGURE 3.1.1.G**) were rescued from the 178 melon fruits set (**TABLE 3.1.2**). Parthenogenetic embryos were rescued in all donor genotypes, the highest number of parthenogenetic embryos found per genotype was 16, in Melito and the lowest 3, in PS-1901. The ratio of parthenogenetic embryos rescued per melon fruit ranged between 0.14 and 0.38. Although all melon fruits carried a normal number of seeds (between 300 and 500) the 71.91% of fruits had no parthenogenetic embryos. The 53 haploid embryos were

recovered from 50 melon fruits. No significant differences (P>0.05) were found using a Chi Square test for the number of parthenogenetic embryos among genotypes.

The parthenogenetic embryos rescued were transferred to solid E20A medium for germination and further plantlet development (**FIGURE 3.1.1.H,I**). From 53 embryos, six failed to germinate, appearing a necrosis in the cotyledonary embryos at the second or third week since rescue. From the 47 germinated embryos, 21 plantlets suffered a stagnation of development and died. No significant differences were found between genotypes for embryo germination and plantlet development (P>0.05). Thus, 26 parthenogenetic independent-genotypes plantlets were able to grow *in vitro* and micropropagation was carried out until greenhouse acclimation (**FIGURE 3.1.1.J**).

TABLE 3.1.2. Parthenogenetic efficiencies of the seven genotypes of melon "Piel de Sapo."

		Parthenogenesis induction with irradiated pollen				Germination and *in vitro* growth		Ploidy-level					
Genotype	**Pollinated flowers**	**% set flowers**	**Melon fruits**	**Embryos**	**Embryos/melon fruit**	**Percentage of mortality**	**Embryos survived**	**n**	**(%)**	**2n**	**(%)**	**n/2n**	**(%)**
PS-1305	165	10,30	17	6	0,35	33.33	4	3	75	1	25	0	0
PS-1901	143	15,38	22	3	0,14	33.33	2	1	50	1	50	0	0
PS-0301	130	24,62	32	12	0,38	75	3	2	66.67	1	33.33	0	0
PS-0709	196	9,69	19	5	0,26	40	3	3	100	0	0	0	0
PS-2001	169	18,34	31	6	0,19	33.33	4	2	50	2	50	0	0
Melito	202	17,82	36	16	0,44	56.25	7	5	71.43	1	14.29	1	14.29
PS-2301	123	17,07	21	5	0,24	40	3	3	100	0	0	0	0
Total	1128	15,78*	178*	53	0,30*	50.94	26	19	73.08	6	23.08	1	3.84

Values followed by * are significantly different ($P<0.05$) between genotypes.

TABLE 3.1.3. Parthenogenetic embryo rescue methods assayed for embryo detection and rescue.

Detection method	Melons opened	Embryos rescued	Ratio of fruits containing embryo
One-by-one	28	10	0.35
X-Ray	127	44	0.34
Liquid medium	23	1	0.04

Values followed by * are significantly different (P<0.05) between methods.

Ploidy-level and chromosome doubling

In order to maximize the number of DH plantlets and to ensure the recovery of seeds from DH genotypes, ploidy-level of the parthenogenetic germinated plantlets was analysed prior to chromosome doubling. Parthenogenesis was found to be successful since haploid, spontaneous DH and mixoploid plantlets were recovered. The ploidy-level of the 26 parthenogenetic lines was analysed by flow cytometry and showed that the 73% were haploid (**FIGURE 3.1.1.M**), the 23% were spontaneous DH (**FIGURE 3.1.1.N**), and one plantlet was found to be mixoploid (**TABLE 3.1.2**). The six spontaneous DH plants were acclimatized in the greenhouse, if they produced pollen and no chromosome doubling was applied. The mixoploid line presented a high ratio of haploid male flowers without pollen and was treated as a haploid.

Chromosome doubling of the 20 haploid parthenogenetic plants was done using different protocols to establish the most efficient one (**TABLE 3.1.4**). Two antimitotic compounds, colchicine and oryzalin, were assayed for *in vitro* chromosome doubling. On the other hand, colchicine was used for *in vivo* chromosome doubling. A total of 114 nodules or shoot tips were treated *in vitro*, 67 and 47, for colchicine and oryzalin, respectively. *In vitro* colchicine treatment resulted in a high number of dead nodules and shoot tips, 86.57%. From the survived plantlets, only two were successfully chromosome doubled and survived the acclimatazion. *In vitro* oryzalin treatment had a lower short-term death, 89.36% of the nodules or shoot tips survived the next two weeks since the chromosome doubling treatment and developed two to three new leaves. Nevertheless, from the 42

survived nodules and shoot tips, 41 presented a high level of hyperhidricity in the base of the nodules or shoot tips that impeded root growing and therefore, no acclimatazion was possible. Final rate of mortality was 95.74%. Only one plantlet was successfully chromosome doubled and acclimatized.

Due to the low values of the chromosome doubling in the *in vitro* treatments, *in vivo* chromosome doubling was assayed. Haploid plantlets were acclimatized in the greenhouse (**FIGURE 3.1.1.K**). *In vivo* chromosome doubling was done using colchicine as the antimitotic agent. A total of 150 plant tips were treated with colchicine and 57.33% survived the treatment. From survived plants, 69.77% remained haploid, 9.30% were mixoploids and 20.93% successfully chromosome doubled. The ploidy of chromosome doubled plants, which was analysed by flow cytometry, was re-checked phenotypically to uphold the successful duplication of plants by checking the presence of pollen in male flower

TABLE 3.1.4. Chromosome doubling protocols assayed.

Treatment					Ploidy-level							
Antimitotic compound	**Concentration ($mg \cdot L^{-1}$)**	**Time (h)**	**Explants or Apical meristems treated**	**Survival (%)**	**n**	**%**	**n/2n**	**%**	**2n**	**%**	**Acclimated plants**	**Melon fruits recovered**
Colchicine *in vitro*	500	12	67	86.57	7	36.84	12	63.16	0	0	2	0
Oryzalin *in vitro*	50	18	47	95.74	1	50	0	0	1	50	1	0
Colchicine *in vivo*	5,000	2	150	42.67	45	70.31	6	9.38	13	20.31	-	12

Parameters followed by * are significantly different ($P < 0.05$) between methods.

DH seed recovery and pollen counts

To recover DH seed, spontaneous DH lines and chromosome doubled lines were self-pollinated (**FIGURE 3.1.1.L**). The 33% of DH lines presented male flowers with pollen together with haploid male flowers without pollen, those plants were classified as phenotypically mixoploid although being detected as pure DH by flow cytometry. No fruit recovery was possible from the *in vitro* chromosome doubled plants. From the *in vivo* duplicated plants, a total of twelve melon fruits were recovered from eight independent parthenogenetic DH lines (**TABLE 3.1.5**). A total of 372 female flowers were pollinated and the fruit set was 3.23%. Genotypes from which no melon fruit was recovered fruit set was impossible. Three out of twelve melon fruits, DH2-PS-2001 and DH8-Melito, carried empty seeds. One chromosome doubled plant, DH4-Melito, did not develop male neither female flowers and no pollination was possible. Finally, DH seed was obtained from six DH plants: DH11-PS-1305, DH3-PS1901, DH9- DH0301, DH5-PS0709, DH10-Melito and DH1-PS-2301.

TABLE 3.1.5. Melon fruits recovered from DH lines.

Doubled haploid genotype	Number of clones	Pollinated flowers	Phenotypical ploidy	Pollen observations	Melon fruits
DH11-PS-1305	5	58	Diploid	Normal male flowers	1
DH3-PS1901	3	47	Diploid	Normal male flowers	2
DH9-DH0301	4	62	Diploid	Small size male flowers with less pollen	1
DH5-PS0709	2	28	Diploid	Normal male flowers	1
DH2-PS-2001	2	36	Diploid	Indehiscent pollen	2
DH8-Melito	3	42	Diploid	Normal male flowers	1
DH10-Melito	3	54	Mixoploid	Normal and haploid male flowers	1
DH1-PS-2301	4	45	Diploid	Normal male flowers	3

DISCUSSION

DH technology has entailed a great progress in plant breeding because of the production of homozygous lines in a shorter time compared to traditional breeding. In Cucurbitaceae, DHs are usually produced for commercial means, either to be used as homozygous stable cultivars or as parental pure lines for hybrid F1 seed production. Thanks to heterosis, hybrid F1 cultivars have enhanced traits than their own parental lines. In this work, the donor material was a batch of seven genotypes of melon 'Piel de Sapo' type evaluated and characterized for their agronomic traits and pathogen resistances. Later on, their parthenogenetic potential was evaluated focusing on pollination with irradiated pollen, parthenogenetic embryo rescue, *in vitro* plantlet performance and chromosome doubling. The production and the consumption of melon 'Piel de Sapo' type is localized mainly in Spain due to cultural reasons where it has a high commercial value because of its differentiated quality. Besides, Spain is the eighth country in terms of melon fruit production worldwide and is the first country in terms of exportation to Europe. We attempted to obtain DH lines of melon with the aim to use them as parental donor lines for commercial hybrid F1 cultivars.

The agronomic traits of melon fruits and the pathogen resistances of the donor material were evaluated in order to analyse the potential use of parthenogenetic-derived DH lines as parental for melon 'Piel de Sapo' hybrid F1 cultivars. The agronomic results showed a great variability of melon fruit parameters between the seven evaluated genotypes. Although Melito cultivar presented low pathogen resistances its melon fruits were valuable because of its small dimensions and the high aroma and sucrose content. Monoecious plants are more likely to have elongated fruits (Robinson, 2000), and PS-0301 genotype was monoecious and presented more elongated fruits in comparison to the other six genotypes. The ºBrix and aroma of melon fruits were not always correlated. Flavor depends upon taste (sweetness and acidity) and aroma. Besides, ºBrix only measures the concentration of predominant sugars, as fructose, sucrose and glucose, and organic acids. Aroma is often considered to play a dominant role in flavor of fruits and vegetables and is dependent upon low-molecular-weight-volatile compounds as largely esters, alcohols, aldehydes and ketones, which are not measured with the refractometer (Kader, 2008). Therefore, melon fruits of PS-1305, PS-1901 and Melito were considered as the best ones in terms of balance between aroma and sugar content. The majority of genotypes assayed presented pathogen resistances. Pathogen

resistance or susceptibility to *Podosphaera xhantii*, causing powdery mildew, *Fusarium oxysporum* f.sp. *melonis*, causing fusarium wilt, and MNSV was evaluated because are the major diseases in melon. The use of resistant cultivars is the best approach to control pathogen spreading and disease. In the southern of Europe, *Podosphaera xhantii* races 1, 2 and 5 are the most frequent (Yuste-Lisbona et al., 2010). Although powdery mildew can be controlled by fungicides its long-term use led to fungicide resistance of powdery mildew. The use of resistant cultivars is a more effective and environmentally safe way to control the disease. On the other hand, *Fusarium oxysporum* f.sp. *melonis* is one of the most difficult diseases to control because the pathogen is soil-borne and remains viable in the soil as chlamydospores (Joobeur et al., 2004). Concerning to MNSV, the best source of resistance in melon is the *nsv* gene, which confers a recessive resistance to MNSV (Nieto et al., 2007). Therefore, genotypes such as PS-1305, PS-0709 and PS-2301, were considered as the best genotype it terms of pathogen resistance.

The genotype of the donor material has a crucial influence for the success of DH protocols as reported in many species, including melon. Parthenogenesis in inodorus 'Piel de Sapo' type genotypes has been reported once and had not been much studied in comparison to other genotypes such as inodorus 'Galia' type, chinensis or cantalupensis, possibly because of its local importance. In this parthenogenetic study, the genotypic response of seven genotypes of melon 'Piel de Sapo' type differed for: fruit set when pollinated with irradiated pollen; parthenogenetic embryo induction; haploid embryo germination; chromosome doubling; and, fruit set of DH lines. The parthenogenetic ability of melon 'Piel de Sapo' germplasm used was lower than other genotypes such as chinensis, cantalupensis or inodorus (Gonzalo et al., 2011; Lim and Earle, 2008; Lotfi et al., 2003).

Fruit set of donor plants after pollination with irradiated pollen is the first step of *in situ* parthenogenesis. In Cucurbitaceae, *in situ* parthenogenesis induction through gamma-ray irradiated-pollen has been achieved in melon, cucumber, watermelon and winter squash since the first report of Sauton and Dumas de Vaulx (1987b). Nevertheless, no reports focus on the efficiency of the pollination with irradiated pollen. In this study, the number of pollinated flowers with irradiated pollen and its later development or drop was recorded. The efficiency of pollination varied between genotypes, the lowest value was 9.69% and the highest 24.62%, in PS-0709 and PS-0301, respectively. The low number of developed female flowers (15.78% in average) and fruit set is attributable to the irradiation process suffered by the pollen. Although irradiated pollen can germinate on the stigma and grow within the style reaching the embryo sac is genetically inactivated to fertilize the egg-cell and the polar nuclei.

Therefore, irradiated pollen stimulates egg-cell division and induces haploid embryos (Cuny, 1992). Pollen sensitivity to irradiation its attributed to radio-resistance, and the viability of pollen is decreased along with the irradiation exposure. Previous reports in melon (Godbole and Murthy, 2012; Gonzalo et al., 2011; Lim and Earle, 2008) used an irradiation exposure of 250 Gy, therefore, prior to the experiment, this dose was evaluated based on fruit set and pollen germination assays (data not shown). Moreover, during pollination with irradiated pollen, fruit set was observed to be dependent on: the time of the year, being August the period when more fruits set; the stage of donor plants, at the beginning of flowering and the end of the greenhouse culture fruit set was low; and, the weather, cloudy pollination days resulted in less fruits than shinny days. Pollen storage viability was evaluated through the pollination with irradiated male flowers with zero, one and two days since irradiation. The storage of irradiated male flowers in plastic pots in darkness for one day was successful to maintain pollen viability. Therefore, pollen could be used to pollinate female flowers. More than one day of storage resulted in a decrease of pollen viability and inability to set melon fruits.

The parthenogenetic embryo production was reported to be genotypic dependent. From all genotypes, a total of 178 melon fruits and 53 embryos were obtained. Normally, the parthenogenetic embryo efficiency is expressed as embryos per seed. Nevertheless, the process of detecting embryos is tedious enough to additionally count the seeds. In this study, the efficiency was expressed as parthenogenetic embryos contained per fruit. The ratio of embryos per fruit in melon 'Piel de Sapo' ranged between 0.14 and 0.44, similar to the 0 to 3 reported in genotypes of 'Piel de Sapo' by Gonzalo et al. (2011). Besides, it was lower than the ratio of 4 to 18 in *inodorus* genotype of the 'Galia' type reported by Lotfi et al. (2003) and the high ratio of 16 reported by Lim and Earle (2008). About one third of melon fruits contained at least one embryo, meaning that the vast majority of melon fruits had an average of 400 empty seeds. The parthenogenetic embryo detection process is laborious and time-consuming, the results are very inefficient compared with the time invested. Because of this, different methodologies have been described to detect parthenogenetic embryos, being the inspection of seeds one-by-one the most commonly applied (Chun et al., 2006; Godbole and Murthy, 2012; Smiech et al., 2008), followed by X-ray radiography of seeds (Claveria et al., 2005; Dolcet-Sanjuan et al., 2004) and the culture of seeds in liquid medium (Lotfi et al., 2003). In this study, three methods were assayed in order to reduce the amount of time and work invested during the process of embryo detection without compromising the embryo itself. Although seed

culture in liquid medium reduced drastically the amount of work it was not effective and compromised the parthenogenetic embryo because of endophytic bacterial and fungi contaminations, despite the initial sterilization of seeds. On the other hand, one-by-one and X-ray methods resulted in a similar ratio of embryo per melon, 0.28 and 0.35, showing that both did not compromise the obtention of embryos. Nevertheless, X-ray method was five time faster than one-by-one method. Then, X-ray method was selected for routine laboratory use.

Once parthenogenetic embryos were detected, they were cultured *in vitro* for germination, development and micropagation. From 53 rescued embryos, 26 germinated, grew *in vitro* and developed plantlets. The 11.32% of embryos failed to germinate and from those germinated, the 39.62% died before the first micropropagation was possible because failed to grow and did not develop the first true leaf. *In vitro* germination and growth are critical steps that can jeopardize the *in situ* parthenogenetic process. Deleterious gene combination in homozygosity regulating vegetative growth may be responsible of hampering germination and plantlet development (Geoffriau et al., 1997). During the *in vitro* process there is a high selection pressure impeding the survival of embryos with deleterious recessive alleles in homozygosity (Cuny, 1992). The results showed a mortality of 25-66.67% depending on the genotype and is in accordance with other authors, reporting a 42-62% (Lim and Earle, 2008) or 62-84% (Lotfi et al., 2003).

To restore diploid chromosome content in haploid melon plants, induced chromosome doubling is mandatory. In cucurbits, the number of spontaneous doubled haploids obtained during DH methodology is usually low compared with other species, that can represent the 30-55% of androgenetic plants in bell pepper (Irikova et al., 2011; Keleş et al., 2015) or 30% in rice (Hooghvorst et al., 2018). The ploidy-level of the produced plants was analysed: 73% were haploid, 4% mixoploid and 23% spontaneous chromosome doubled. The ploidy-level results presented are in accordance with those of Lim and Earle (2008), who found a 73% of haploids and 27% mixoploids in melon; or Kurtar and Balkaya (2010), that produced 76.71% spontaneous DHs and 23.29% haploids in squash; or, Sauton (1988, 1989), who reported spontaneous doubling in cucumber and melon for the first time. Spontaneous duplication can occur when endomitosis or nuclear fusion happens. In endomitosis process, cell multiplicate chromosomes and separate them in each cell pole during early mitosis, nevertheless, cell fails to divide, and two sets of chromosomes is restituted. In nuclear fusion, two or more synchronized nuclei divide and develop a common spindle (Kasha, 2005). Spontaneous DHs and mixoploids have an endomitotic or

nuclei fusion origin. Notwithstanding, in spontaneous DHs, the endomitosis or nuclear fusion took place at early stages of the development of the egg-cell, and in mixoploid plants occurred later, causing a different ploidy-level of the germ cells. Then, induced chromosome doubling of haploid plants is necessary prior to DH seed recovery. Colchicine is the most used antimitotic for chromosome doubling in DH technology. When haploids are treated with antimitotic compounds a so-called C-mitosis can take place. During interphase, cells have their chromosomes duplicated with the chromatid sisters placed in each pole of the cell bound by the centromere' spindle tubule. The antimitotic compound interacts with the tubulin subunits and destabilize the spindle tubule arresting cells during interphase. Chromosome doubling is a required step in parthenogenesis DH protocols in Cucurbitaceae. Three different protocols were assayed for chromosome doubling, two protocols *in vitro* with colchicine or oryzalin, and one *in vivo* with colchicine. *In vitro* protocols resulted in a high mortality rate due to antimitotic toxicity. The recorded chromosome duplication efficiency of 500 $mg \cdot L^{-1}$ of colchicine for 12 h *in vitro* treatment was 63.16%. However, majority of plantlets failed to develop after the treatment and before the ploidy-level analysis. The rate of chromosome doubled and successfully acclimatized plants was 2.98% (two plants). Similar results were recorded for 50 $mg \cdot L^{-1}$ of oryzalin for 18 h *in vitro* treatment which resulted in one plant (2.13%) survived and chromosome doubled. Those results show a high sensitivity of the genotypes to the antimitotic compounds and to the *in vitro* culture once treated, which do not line with other reported studies that show *in vitro* chromosome doubling treatment as successful and the preferred (Dong et al., 2016; Ebrahimzadeh et al., 2018). This could be explained as a recalcitrant performance of the 'Piel de Sapo' genotypes not only to the antimitotic treatment but also to *in vitro* culture, which in turn resulted in a low production of DH lines. Due to the low efficiency of survival and chromosome doubling of *in vitro* treatments, *in vivo* 5000 $mg \cdot L^{-1}$ of colchicine for 2 h on apical meristems was assayed. Resulting in 69.77% of survival and 13.33% of chromosome doubling, being 8% DHs and 5.33% mixoploids. Other authors have reported higher chromosome duplication efficiencies when treating *in vivo* with colchicine as 46.03% in 'Kirkagac and ''Yuva Hasanbey' melon genotypes (Solmaz et al., 2011) or 19% (Lim and Earle, 2008). The *in vivo* chromosome doubling efficiencies were acceptable in spite of being low, those are in line with the low *in vitro* efficiencies and other parameters analysed previously, which support the hypothesis of the recalcitrant performance of 'Piel de Sapo' genotypes during the entire parthenogenetic process. The fruit set of the 20 DH lines and the eight mixoploid lines was low. A total of 12 fruits from eight independent parthenogenetic

DHs were recovered. Pollination of induced chromosome doubled plants was dramatically difficult, an average of 3.23% of pollinated female flowers set fruit. This ratio was even lower than the pollination with irradiated pollen. From the fruits recovered, both of DH2-PS-2001 and the one of DH10, contained the usual amount of seeds despite all of them were empty. Consequently, DH seed was not recovered from those genotypes. Lim and Earle (2008) proved that pollen viability of chromosome doubled plants its usually low affecting fruit set and seed viability. In their study, they recommend *in vitro* chromosome doubling because had higher pollen germination rates than *in vivo* chromosome doubled plants. We had no success in *in vitro* chromosome doubling, and the *in vivo* chromosome doubled plants had a low capacity to set fruits.

In this study, 'Piel de Sapo' donor material had traits with potential value for commercial purposes such as melon fruit morphology, sweetness and aroma, and pathogen resistances against important diseases such as powdery mildew, fusarium wilt and MNSV. Moreover, the *in situ* parthenogenetic ability of 'Piel de Sapo' germplasm was evaluated, showing: a low capacity of fruit set when pollinated with irradiated pollen, a low production of parthenogenetic embryos, a poor *in vitro* culture performance, a low chromosome doubling and a low fruit set of DH lines once chromosomes were doubled. The 'Piel de Sapo' *inodorus* type can be considered as a recalcitrant genotype for parthenogenesis in melon species in comparison to other genotypes. Nevertheless, we succeed to obtain DH seed that have a great value for hybrid F1 seed production and commercialization. During the parthenogenetic process, X-ray method was concluded as the most successful and optimum method to detect and rescue parthenogenetic embryos. The poor performance of 'Piel de Sapo' genotypes showed during *in vitro* culture could be enhanced changing media composition instead of using the traditional E20A medium. In addition, an *in vivo* chromosome doubling method with colchicine was adapted and resulted as the most successful for chromosome doubling of haploid plants, in front of *in vitro* chromosome doubling methods with colchicine or oryzalin. Although parthenogenetic DH plants were obtained from six out of seven melon 'Piel de Sapo' genotypes further improvements of the process using variations should be assayed in order to produce a higher number of DH plants that could be used in melon breeding programs.

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CHAPTER II. Efficient knockout of phytoene desaturase gene using CRISPR/Cas9 in melon

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ABSTRACT

CRISPR/Cas9 system has been widely applied in many plant species to induce mutations in the genome for studying gene function and improving crops. However, to our knowledge, there is no report of CRISPR/Cas9-mediated genome editing in melon (*Cucumis melo*). In our study, phytoene desaturase gene of melon (*CmPDS*) was selected as target for the CRISPR/Cas9 system with two designed gRNAs, targeting exons 1 and 2. A construct (pHSE-CmPDS) carrying both gRNAs and the Cas9 protein was delivered by PEG-mediated transformation in protoplasts. Mutations were detected in protoplasts for both gRNAs. Subsequently, *Agrobacterium*-mediated transformation of cotyledonary explants was carried out, and fully albino and chimeric albino plants were successfully regenerated. A regeneration efficiency of 71% of transformed plants was achieved from cotyledonary explants, a 39% of genetic transformed plants were successful gene edited, and finally, a 42-45% of mutation rate was detected by Sanger analysis. In melon protoplasts and plants most mutations were substitutions (91%), followed by insertions (7%) and deletions (2%). We set up a CRISPR/Cas9-mediated genome editing protocol which is efficient and feasible in melon, generating multi-allelic mutations in both genomic target sites of the *CmPDS* gene showing an albino phenotype easily detectable after only few weeks after *Agrobacterium*-mediated transformation.

Keywords: CRISPR/Cas9, knockout, mutation, phytoene desaturase, melon.

INTRODUCTION

Genome editing tools have the potential to modify genomic sequences with accuracy. Some of these tools are: homologous recombination (HR), targeted induced local lesions in the genome (TILLING), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), or clustered regularly interspaced short palindromic repeats associated to nuclease Cas9 (CRISPR/Cas9). ZFN, TALENs and CRISPR/Cas9 are site-specific nucleases. The CRISPR/Cas9 genome editing tool was developed in 2013, and in comparison to other genome editing tools has better efficacy, efficiency, versatility and is simpler (Bortesi and Fisher, 2015).

CRISPR/Cas9 system cleaves a specific region of DNA by the Cas9 nuclease, which is guided by a 20-nt sequence named RNA-guide (gRNA). The association between Cas9 and gRNA, and the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA (typically NGG), allows a precise editing of DNA target sequences. The endonuclease domain induces DNA double-strand breaks (DSB), which can be repaired by either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) generating insertions and deletions events (INDELs) and substitutions (Shan et al., 2014; Symington and Gautier, 2011). The major uses of CRISPR/Cas9 in plants have been gene knockouts to elucidate the function of a target gene by gene mutation and transcriptional regulation (Liu et al., 2017). This application allows genes function studies, to knock out genes that negatively affect food quality, to confer resistance to pathogens or divert metabolic flux away from valuable end-products (Bortesi and Fisher, 2015).

CRISPR/Cas9 system is theoretically applicable to all plant species, but many of them lack the experimental demonstration of its applicability. Since 2013, CRISPR/Cas9 has been applied in *Oryza sativa* (Shan et al., 2013), *Arabidopsis thaliana* (Feng et al., 2013), *Nicotiana benthamiana* (Nekrasov et a., 2013), *Solanum lycopersicum* (Brooks et al., 2014), *Zea mays* (Liang et al., 2014) and soybean (Jacobs et al., 2015), among other species. The strategy to mutate and knockout the phytoene desaturase gene (*PDS*) by CRISPR/Cas9 has been widely applied to quickly demonstrate the feasibility of CRISPR/Cas9 since its mutation causes photobleaching or albino phenotype. Through transient expression assays or transformation methods, CRISPR/Cas9 mutations have been demonstrated and studied in plants. Within the Cucurbitaceae family, this genome editing technique has only been

reported as successfully applied in cucumber (Chandrasekaran et al., 2016) and watermelon (Tian et al., 2017, 2018).

Melon (*Cucumis melo*) belongs to Cucurbitaceae and is an important plant because of its specific biological properties and economic value of its fruit. In fact, 31 million tons of melon were produced worldwide in 2016 (FAO, 2016), being an important crop in Mediterranean and East Asian countries. The recent melon genome publication by Garcia-Mas et al. (2012) and the versatility of CRISPR/Cas9 allows to study and explore gene functions in melon. However, to our knowledge, CRISPR/Cas9 application in melon has not been reported. Therefore, the aim of this study was to demonstrate, for the first time, the applicability of CRISPR/Cas9 system on melon by performing a gene knockout of the melon phytoene desaturase gene (*CmPDS*) in protoplasts and plants.

Vector construction

Construct for constitutive expression of Cas9 was done following the protocol of Xing et al. (2014). The binary vectors pHSE401 and pCBC-DT1T2 (Addgene plasmids #62201 and #50590, respectively) were a gift from Qi-Jun Chen. For the assembly of two gRNAs into pHSE401, a four-primer mixture with DT1-F0-PDS/DT2-R0-PDS and DT1-BsF-PDS/DT2-BsR-PDS in a proportion 1:20 (**TABLE 3.2.1**), were used for PCR amplification along with pCBC-DT1T2 and Phusion High-Fidelity DNA Polymerase (NEB) following the manufacturer's recommendations. PCR protocol was 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min and a final extension at 68 °C for 5 min. PCR product DT1T2-PCR (626 bp) was separated on 2% agarose gel and agarose purified with PureLink Quick Gel Extraction Kit (Invitrogen). Then, DT1T2-PCR product was assembled into pHSE401 by Golden Gate cloning method, using BsaI and T4 Ligase (NEB) following the manufacturer's recommendations. The binary vector constructed, named as pHSE-CmelPDS (**FIGURE 3.2.1.C**), containing both guides, gRNA1 and gRNA2, was used to transform NEB 5-alpha Competent E. coli (High Efficiency; NEB). Positive clones were confirmed by Sanger sequencing, plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and finally transformed into *Agrobacterium tumefaciens* EHA105.

TABLE 3.2.1. Analysis of gRNA sequences used for CRISPR/Cas9 vector construction.

	Nucleotide sequence (5' - 3')	**Exon and position**	**PAM**	**GC (%)**	**Mutation rate (%)**	
					Protoplast	**Plants**
gRNA1	TAGTGAGATTGTGGGCGAT	Exon 1; 123-141	GGG	47.39	25	45
gRNA2	TAGACCACAGATAGATGAT	Exon 2; 21-39	GGG	36.84	25	42

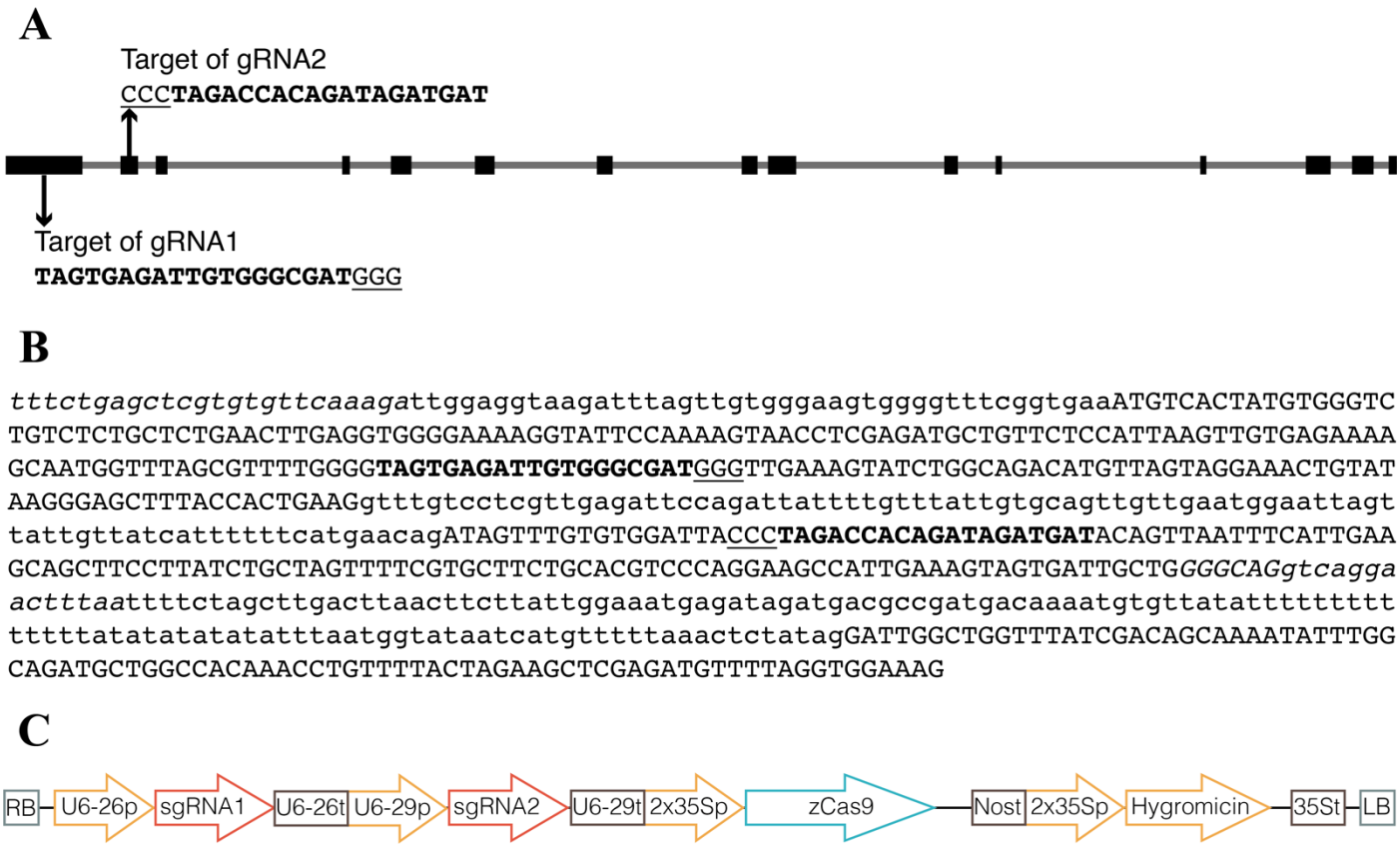

FIGURE 3.2.1. Schematic representations of the melon *CmPDS* target gene (MELO3C017772.2 in ICUGI data base), location of the gRNA1 and gRNA2, and the CRISPR/Cas9 vector. (**A**) Schematic representation of melon *CmPDS* gene with two target sites indicated as bold and PAM sequences as underlined. (**B**) Representation of *CmPDS* target sequences. Exons are shown as capital letters, introns as lowercase, gRNA1 and gRNA2 as bold, PAM sequences as underlined, and primers used for gene sequencing as italics. (**C**) Schematic representation of the CRISPR/Cas9 binary vector used for melon transformation. Arabidopsis thaliana promoters and terminators drive expression of gRNA1(AtU6-26p and AtU6-26t) and gRNA2 (AtU6-29p and AtU6-29t). The Cauliflower mosaic virus promoter (*CaMV 35S*) drives the expression of the Cas9 gene.

Extraction of melon protoplast and its transformation

Melon protoplasts were extracted and transfected following the protocol from Yoo et al. (2007) with minor modifications. 10-15-days old melon cotyledons were chopped with a razor blade and digested with 10 mL of enzyme solution (20 mM MES, 1.5% cellulase R10, 0.4% macerozyme R10, 0.4 M mannitol, 20 mM KCl and pH 5.7) and incubated in an orbital shaker for 3 h at 24 °C and 70 rpm. Then, the protoplast solution was collected on ice into a 15 mL tube and diluted up to 10 mL with W5 solution (2 mM MES, 0.5 M mannitol, 20 mM KCl and pH 5.7). The protoplast solution was filtered through a 75-µm mesh, and protoplasts were collected by centrifuging at 100 g for 5 min at 4 °C. The supernatant was discarded and protoplasts were resuspended up to

10 mL of W5. Protoplasts were kept on ice for 30 min, the supernatant was discarded, and the protoplast solution was adjusted to a concentration of $2 \cdot 10^5$ protoplasts·mL^{-1} with the help of a hemocytometer with MMG solution (4 mM MES, 0.4 mannitol, 15 mM $MgCl_2$ and pH 5.7). For the transformation, 10 μL of pHSE-CmelPDS vector (20-30 μg) were mixed with 100 μL of protoplasts ($2 \cdot 10^4$) and 110 μL of PEG-calcium transfection solution (40% PEG4000, 0.2 M mannitol, and 100 mM CaCl2), and incubated for 15 min at 25 °C in darkness. Transfection was stopped by adding 400 μL of W5 supplemented with 5 mg·L^{-1} hygromycin and cultured in darkness for 24 h at 24 °C. The transfected protoplasts were then collected for genomic DNA extraction.

Agrobacterium-*mediated transformation and plant regeneration*

Cucumis melo var. *cantaloupensis* inbred line "Charentais" (provided by ROCALBA S.A.) was used as the source of explants for genetic transformation. Seed coats were removed, and seeds were surface-sterilized with a 20% sodium hypochlorite solution containing 8 drops·L^{-1} of Tween 20 for 10 min in agitation and rinsed two times with sterile distilled water. Seedlings were pre-cultured on MS medium supplemented with 3% sucrose for two days. Then, nodal cotyledons were used as explants after removing the embryo. Explants were vacuum-infiltrated with *Agrobacterium tumefaciens* EHA105 infection solution for 5 min twice and an additional 5 min in a shaker without vacuum. To prepare the *Agrobacterium tumefaciens* EHA105 infection solution, a single colony containing the selected binary CRISPR/Cas9 vector was picked and put in a 5 mL starter culture of LB containing 50 mg·L^{-1} Kanamycin and 100 mg·L^{-1} Rifampicin and cultured for 20 h at 24 °C at 130 rpm. Next, 1 mL of the starter culture was transferred to 45 mL LB culture containing Kanamycin and Rifampicin and cultured until OD600 reached 0.6. Once the *Agrobacterium* transformation was carried out, explants were transferred to co-cultivation medium consisting on MS medium supplemented with 3% sucrose and cultured 48 h at 24 °C in the dark. Afterward, explants were rinsed with antibiotic solution composed of 500 mg·L^{-1} Cefotaxime and 300 mg·L^{-1} Ticarcillin in distilled water. Then, the explants were transferred into selective medium which was MS supplemented with Sucrose 3%, 2.22 μM BAP, 0.48 μM IAA, 4 μM $CuSO4 \cdot 5H_2O$, 500 mg·L^{-1} Cefotaxime, 300 mg·L^{-1} Ticarcillin and 5 mg·L^{-1} hygromycin B. Every two weeks explants were transferred to fresh selective media. Regenerated plants were transferred to E20A (Sauton and Dumas, 1987) media supplemented with 5 mg·L^{-1}

hygromycin B. Explants on selective medium and plants were cultured under a 16/8h light/dark photoperiod at 24 °C.

Detection of transgene and CRISPR/Cas9 mutation

Genomic DNA of protoplasts and plants was extracted following the methodology of Doyle and Doyle (1987) with minor modifications. The transgene presence in plant (**FIGURE 3.2.1.A**) was confirmed by PCR using specific primers named pHSE401.SeqF and pHSE401.SeqR (**TABLE 3.2.1**). A fragment flanking gRNA1 and gRNA2 (846 bp) of the *CmPDS* gene from transgenic plants and transformed protoplasts was amplified using F-CmPDS and R-CmPDS primers, and the amplified PCR product was gel-purified and cloned into pCR-Blunt II-TOPO vector (Life Technologies). Colonies were Sanger-sequenced using M13F and M13R primers for detecting specific additions, deletions and substitutions. Mutation rate was calculated as the ratio of mutated clonal colonies versus total sequenced colonies.

TABLE 3.2.2. Primers used in this study to assemble two gRNAs in the pHSE401 vector, for transgene detection and for gene amplification and sequencing of *CmPDS* gene.

Primer name	**Primer sequence (5' - 3')**
Assembly of two gRNA	
DT1-BsF-PDS	ATATATGGTCTCGATTGCGAGATGCTGTTCTCCATTGTT
DT1-F0-PDS	TGAATGGAGAACAGCATCTCGGTTTTAGAGCTAGAAATAGC
DT2-R0-PDS	AACTAGACCACAGATAGATGATACAATCTCTTAGTCGACTCTAC
DT2-BsR-PDS	ATTATTGGTCTCGAAACTAGACCACAGATAGATGATC
Detection of transgene	
pHSE401.SeqF	TCTTCAAAAGTCCCACATCGC
pHSE401.SeqR	AACCCCAGAAATTGAACGCC
CmPDS gene amplification	
F-CmPDSgRNA1	CTGAGCTCGTGTGTTCAAAGA
R-CmPDSgRNA2	TTAAAGTTCCTGACCTGCCC

Target selection and vector construction for CRISPR/Cas9 system

To test the efficacy of CRISPR/Cas9 system in melon, we chose to disrupt phytoene desaturase gene of *Cucumis melo* (*CmPDS*) which has a single copy, located in the chromosome 7, with reference MELO3C017772.2 in ICUGI data base. The genomic sequence of this reference is 10,443 bp in size, with 14 exons (**FIGURE 3.2.1.A**). Target sites in *CmPDS* were designed using Benchling and two target sites were selected: gRNA1 and gRNA2, in exon 1 and 2 respectively (**FIGURE 3.2.1.A**). Both gRNAs were cloned into one binary vector (pHSE-CmelPDS) carrying the promoter for the Cas9 gene (*CaMV 35S*), the Cas9 gene, AtU6-26p and AtU6-29p promoters and gRNA scaffold (**FIGURE 3.2.1.C**). Potential off-target sites were searched using CRISPR-OFFinder (Zhao et al., 2017), and no potential off-target were detected with 0 to 2 mismatches (Hahn and Nekrasov, 2019) (**TABLE 3.2.1**).

Targeted mutagenesis in melon protoplasts

CRISPR/Cas9 vector pHSE-CmelPDS was tested in protoplasts to validate the functionality of Cas9 via transient expression by PEG-mediated protoplast transfection. A total of 24 protoplast cell colonies were Sanger-sequenced. According to the number of mutated colonies detected, the target efficiency for pHSE-CmelPDS in melon protoplasts was 25% for both gRNAs. Moreover, most of the mutations analysed were substitutions and two nucleotides insertions (**FIGURE 3.2.3** and **TABLE 3.2.3**). These results suggested that the vector was viable to be used in melon for plant regeneration

TABLE 3.2.3. Summary of the alleles, number of colonies and frequency of each allele (%) found in protoplast cells PEG4000-mediated transformed with the CRISPR/Cas9 vector. i: insertions; s: substitution; WT: wild-type.

Alleles found	nº colonies	Frequency (%)
3s/2s	1	4.17
WT/1s	1	4.17
1s/1s	1	4.17
1s/WT	1	4.17
1s/1i	1	4.17
1s/1i	1	4.17
1s/3s	1	4.17
WT/WT	17	70.83

PDS-edited plants phenotype

Alteration of *CmPDS* gene expression function was manifested as albino and dwarf plants (**FIGURE 3.2.2**). Some cotyledons only regenerated albino shoots, meanwhile others regenerated green shoots and after a while exhibited mosaicism or regenerated secondary albino shoots. Complete albino plants exhibited a high level of dwarfism, having a reduced leaf area, a growth lesser than 0.5 cm, and impossibility to perform *in vitro* propagation. A total of 77 plants were regenerated from 958 *Agrobacterium*-mediated transformed cotyledonary explants. The 22.07% of the regenerated plants showed albino phenotype, 4 complete albino plants and 13 chimeric albinism were detected. Albino plants did not survive more than three months after regeneration.

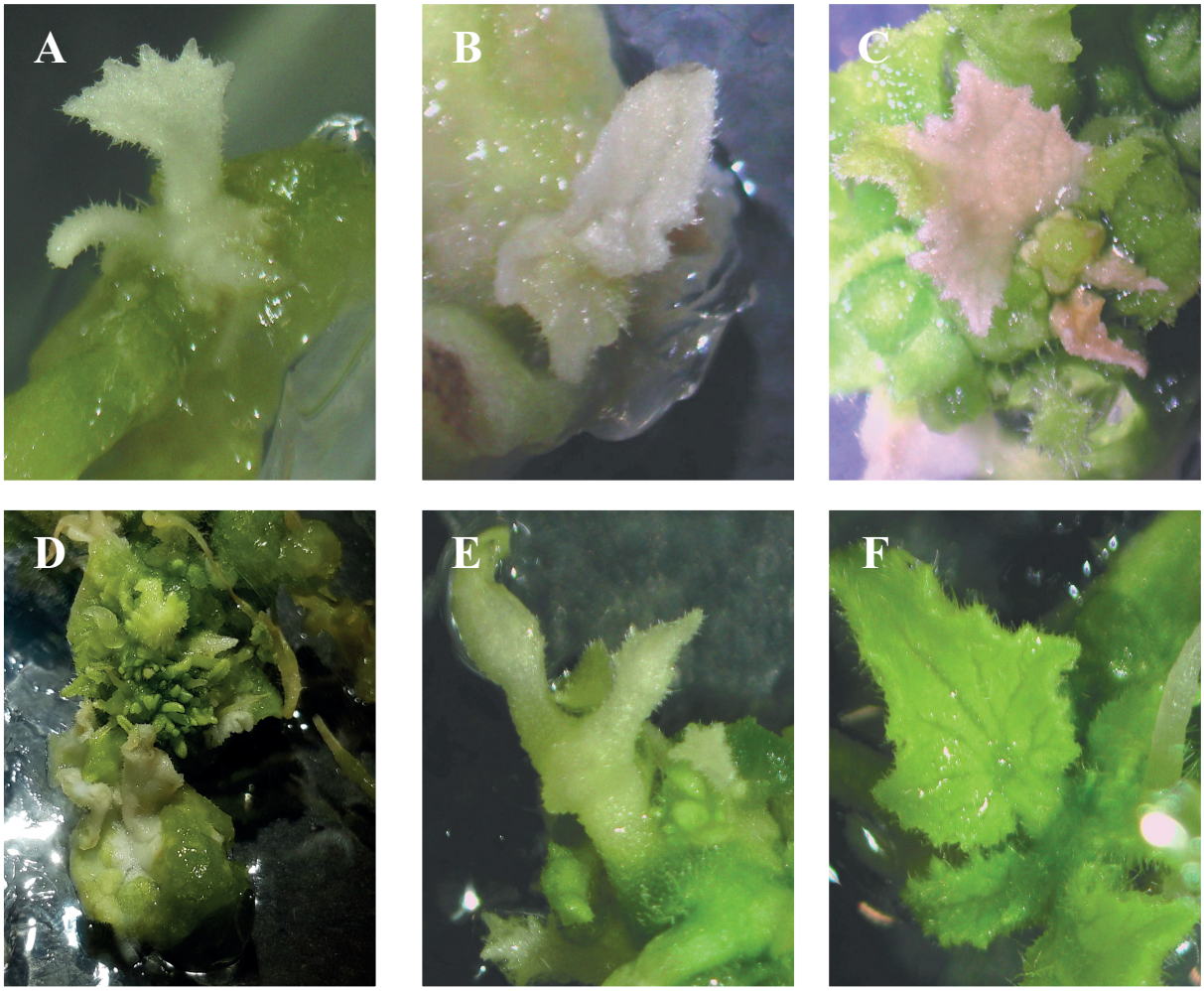

FIGURE 3.2.2. Phenotypic diversity of regenerated CRISPR/Cas9 mutated plants. **A** and **B** panels show fully albino and dwarf melon plants; **C**, **D** and **E** panels show chimeric albino plant presenting a mixture of green and white tissues; and F panel, shows a green WT regenerated plant.

Targeted mutagenesis in transgenic melon plants

Polymerase chain reaction (PCR) was performed in genomic DNA of the regenerated plants to detect the presence of the transgene and to amplify the target gene sequences. Genomic DNA was extracted from 62 plants. A 239 bp fragment within the LB and RB regions was amplified to confirm the presence of transgene. The transformation efficiency on the regenerated plants was 71%. A 502 bp fragment of the *CmPDS* gene

corresponding to the first and second exons together was amplified, and a mutation analysis was performed with 10 plants showing a clearly *PDS*-edited phenotype (**FIGURE 3.2.2**).

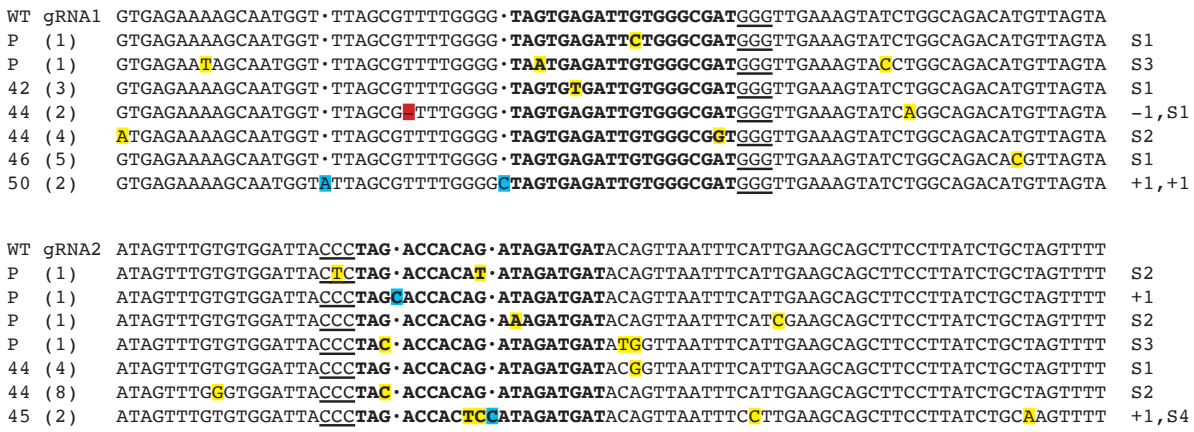

FIGURE 3.2.3. Targeted mutations detected in melon plants and protoplasts induced by CRISPR/Cas9 in each gRNA. Target sequences of *CmPDS* are bolded and the protospacer adjacent motif (PAM) in italics. Aligned sequence data is shown for four plants (number) and protoplasts (P). Number of colonies sequenced are indicated in the left between parentheses. Substitutions are highlighted in yellow; insertions are highlighted in blue, whereas a · is used in sequences with no insertion to maintain the reading frame; and, deletions are highlighted in red.

A variety of mutations was detected in the genotyped plants, including insertions, deletions and substitutions (**FIGURE 3.2.3** and **TABLE 3.2.3**). In 113 sequenced colonies from albino phenotype plants analysing both gRNAs, the target mutation efficiency was 45% and 42% for gRNA1 and gRNA2, respectively. Up to 91% of mutations were substitution events, 7% were insertions, and 2% deletions. Further analysis of the *CmPDS* gene mutations revealed substitutions, insertions and deletion events upstream and downstream of the target region for both gRNAs. Multiple alleles were found in all albino and chimeric plants, showing a high level of chimerism for both target sequences (**TABLE 3.2.3** and **TABLE 3.2.4**). In silico analysis of codon change due to substitutions in plants revealed that 28.56% of substitutions caused an introduction of a STOP codon, 60.71% caused a change in amino acid (AA) codon, and a 10.71% caused no AA codon change (**TABLE 3.2.5**).

TABLE 3.2.4. Summary of the genotyping of ten plants with albino phenotype. Type of mutation of each allele found, number of colonies, frequency of each allele, edition profile of the plant and phenotypic appearance. d: deletions; i: insertions; s: substitution; WT: wild-type.

Plant number	Sequenced colonies	Type of mutation	Frequency (%)	Edition profile	Phenotypic appearance
33	2	3s/WT	22.22	Heterozygous	Chimeric
33	7	WT/WT	77.78		
40	7	1s/1s	77.78	Chimeric	Chimeric
40	1	1s/WT	11.11		
40	1	WT/WT	11.11		
42	3	1s/WT	23.08	Heterozygous	Chimeric
42	10	WT/WT	76.92		
44	2	1d1s/1s	13.33	Chimeric	Albino
44	4	2s/1s	26.67		
44	8	WT/2s	53.33		
44	1	WT/WT	6.67		
45	3	WT/1i4s	25.00	Chimeric	Albino
45	9	1s/WT	75.00		
46	5	1s/WT	33.33	Heterozygous	Albino
46	2	WT/2s	13.33		
46	2	WT/1s	13.33		

46	6	WT/WT	40.00		
49 49	3 8	WT/1s WT/WT	27.27 72.73	Heterozygous	Chimeric
50 50	10 2	1s/WT 2i/WT	83.33 16.67	Heterozygous	Chimeric
59 59	6 2	1s/WT 2i/WT	75.00 25.00	Heterozygous	Chimeric
60 60	7 2	1s/1s WT/WT	77.78 22.22	Heterozygous	Chimeric

TABLE 3.2.5. *In silico* analysis of CRISPR/Cas9 induced substitutions in plants. Nucleotide substitution could result in: introducing a STOP codon, causing a new amino acid (AA) codon, or, no change in the amino acid codon.

Total number of substitutions	**Nucleotide substitutions introducing STOP codon**	**Nucleotide substitutions causing AA change**	**Nucleotide substitutions not causing AA change**
28	28.58%	60.71%	10.71%

DISCUSSION

To date, CRISPR/Cas9 system has been applied for basic research and trait development in many plant species (Karkute et al., 2017; Pennisi, 2013). To our knowledge, this study reports for the first-time gene editing by CRISPR/Cas9 in melon (*Cucumis melo*). We employed this system to target the *PDS* gene in melon, a key enzyme in the carotenoid biosynthesis pathway involved in at least 20 metabolic pathways, including the inhibition of many genes in carotenoid, chlorophyll, and GA biosynthesis pathways (Qin et al., 2007). Successful disruption of *PDS* gene results in a generation of mutants expected to be photobleached or albino, which allows to phenotypically identify the feasibility of CRISPR/Cas9 in melon. Alteration of *PDS* was chosen as a visual marker to easily detect the CRISPR/Cas9 genome editing in melon. In order to improve our chances of success we designed two gRNAs and adapted an existing genetic transformation system in cucumber (Li et al., 2017). Chimeric plants, showing albino and green tissue in the same plant or leaf, and albino plants were regenerated from melon cotyledonary explants, as reported in many other species (Gao et al., 2017; Odipio et al., 2017; Charrier et al., 2019; Pan et al., 2016; Li et al., 2013). A total of 4 fully albino plants and 13 albino chimeric plants were regenerated due to the loss-of-function of *CmPDS* gene.

Most of the mutations analysed in melon protoplasts were substitutions and two insertions were found. Mutations in transgenic melon plants were mostly substitutions, with some deletion and insertion events. Although INDELs are the most common mutations induced by NHEJ, other authors have found similar results concerning the high level of substitutions induced by CRISPR/Cas9. For example, in soybean protoplasts and plants, a high level of substitutions was analysed in the mutations events (Sun et al., 2015); in protoplasts of cotton, only substitutions were detected (Chen et al 2017); in cassava plants, substitutions occurred more frequently than INDELs mutations (Odipio et al 2017); and, in rice the 25-45% of the analysed mutations were substitutions (Macovei et al., 2018).

Agrobacterium-mediated transformation is the only reported method for genetic transformation in melon. One of the major issues when transforming species of the Cucurbitaceae is the occurrence of "escapes", which are non-transgenic well-developed shoots regenerated into selective media. In melon, the regeneration rates from cotyledonary explants are usually high (40-70%), nevertheless, transformation rates of the regenerated shoots are

usually very low, and is highly genotypic dependent (Li et al., 2017; Dong et al., 1991). Melon is a recalcitrant crop for transformation (Dong et al., 1991). In our study, 8% of the cotyledonary explants regenerated shoots, among which a high percentage (71%) proved to be transgenics. Although we did not find biallelic mutations in melon plants and albino plants failed to grow *in vitro*, mutations should be stably inherited in the T1 and T2 generations (Feng et al., 2014). The lethal phenotype of *PDS* mutant plants could be alleviated by optimizing the medium. The use of an additional medium to grow albino plants, increasing sugar composition or increasing cytokinin proportions could allow the survival of albino plants. Furthermore, plant regeneration from cotyledonary explants can induce endoduplication phenomenon that generate tetraploid regenerants (Guis et al., 2000). In this experiment we did not analyse the ploidy of the regenerated albino plants because majority of albino plants were also dwarf hence plant material availability was low.

The pHSE-CmelPDS vector contained the Cas9, a gRNA scaffold and both gRNA guides. Promoters from the cauliflower mosaic virus (*CaMV35S*) and *Arabidopsis thaliana* (U6 and U3), were used for driving the constitutive expression of Cas9 and both sgRNA (gRNA1 and gRNA2), respectively. Within dicotyledons, when *CaMV35S* promotor is used the mutation rate depends on the species, from 20 to 100% (Pan et al., 2016; Jiang et al., 2013). High transcription levels of Cas9 protein and sgRNA are essential for the activity of the CRISPR/Cas9 system (Mao et al., 2017). The expression driver of Cas9 gene is an important factor that can increase the mutation frequency, and the use of a endogenous promoter ensures a high level of transgene expression and allows to increase mutation efficiencies 2 to 7 fold, as showed in soybean and liverwort (Sun et al., 2015; Sugano et al., 2014). The endogenous U6 promoter for sgRNA expression has a key role in the CRISPR/Cas9 efficiency too (Yan et al., 2015). The regeneration efficiency of mutant plants from cotyledonary explants (1.8%) was very similar to those reported in watermelon (Tian et al., 2017). The mutation rate of the sequenced colonies was 42-45% in the analysed transgenic plants, which is lower than the ones found in other dicotyledons. In protoplast transfected cells, mutation was successful despite low edition frequencies, 25% for each gRNA. In comparison to other species this value is low and could be due either to low transfection efficiency or to a lack of CRISPR/Cas9 editing activity. It is possible that the fact that the promoters used were not endogenous is the cause of the efficiency drop of the CRISPR/Cas9 system. Overall, the use of endogenous

promoters for Cas9 and sgRNA expression is the best way to increase the CRISPR/Cas9 efficiency in melon and other cucurbits.

Efficient gene editing in melon presents the possibility to study new gene functions for basic research, and new opportunities for melon productivity by improving biotic stress, melon production and the post-harvest utilization. Towards this, we demonstrated the occurrence of targeted mutagenesis with the CRISPR/Cas9 system in melon protoplasts and plants. The *CmPDS* knockout system described generates easily detectable albino and dwarf plants and mutation events in a frame of 6–8 weeks. Therefore, it provides a valuable method to facilitate rapid assessment and optimization of CRISPR/Cas9 and other genome-editing technologies in melon.

AKNOLEDGMENTS & AUTHORS CONTRIBUTION

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IH designed the experiment, obtained the gene edited plants, interpreted the sequencing data and wrote the manuscript. CL-C obtained the sequencing data and edited the manuscript. SN supervised the experiment.

The authors declare no competing interests.

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.OTHER ARTICLES RELATED TO THE THESIS

CHAPTER III. Chromosome Doubling Methods in Doubled Haploid and Haploid Inducer-Mediated Genome-Editing Systems in Major Crops

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This chapter has been included as part of the RESULTS and GENERAL DISCUSSION of this thesis because of its content and nature.

ABSTRACT

The doubled haploid technique aims to generate pure inbred lines for basic research and as commercial cultivars. The doubled haploid technique first generates haploid plants and is followed by chromosome doubling, which can be separated in time or overlapped, depending the procedure for each species. For a long time, much effort has been focused on haploid production via androgenesis, gynogenesis or parthenogenesis. The obtention of haploid plants has frequently required more optimization and has lagged behind research and improvements in chromosome doubling methods. Nevertheless, chromosome doubling has recently been of renewed interest to increase the rates and efficiency of doubled haploid plant production through trialing and optimizing of different procedures. New antimitotic compounds and application methods are being studied to ensure the success of chromosome doubling once haploid material has been regenerated. Moreover, a haploid inducer-mediated CRISPR/Cas9 genome-editing system is a breakthrough method in the production of haploid plant material and could be of great importance for species where traditional haploid regeneration methods have not been successful, or for recalcitrant species. In all cases, the new deployment of this system will demand a suitable chromosome doubling protocol. In this review, we explore the existing doubled haploid and chromosome doubling methods to identify opportunities to enhance the breeding process in major crops.

Plant breeding has attempted over many decades to increase crop yield and improve cultivar traits. The purpose of plant breeding is to boost agronomical traits such as: disease and insect resistance; tolerance to abiotic stresses like drought, extremes of temperature and salinity; and to increase yield while at the same time enhancing or maintaining nutritional quality.

Conventional breeding by backcrossing is a method to improve an elite line by adding a new trait. By crossing the elite line with a donor line, carrying the trait of interest, an F1 hybrid is obtained and backcrossed with the elite line again. The subsequent offspring is recurrently backcrossed with the elite line again, until the 5th to 8th generations. The final backcrossed line contains the new trait of interest and the characteristics of the elite line, and the genotypic background of the donor line has been cleared (Forster et al., 2007). Backcrossing is effective, but obtaining a stabilized line with the trait of interest is extremely time consuming due to the number of generations needed to be crossed and evaluated (**FIGURE 3.3.1**). Instead, the emergence of doubled haploid (DH) technology in the second half of the 20th century has dramatically reduced the time required to generate pure homozygous lines.

Doubled haploid lines are pure and genetically homozygous individuals produced when spontaneous or induced chromosome duplication of haploid cells occurs. DHs are one of the leading achievements in plant breeding because completely homozygous plants can be produced within a year. DH production includes two major steps: haploid induction and chromosome doubling. Haploid induction attempts to regenerate haploid or spontaneous DH plants, which can be achieved by androgenesis, gynogenesis or parthenogenesis, depending on the species. The chromosome-doubling step is mandatory when spontaneous DHs are not regenerated and is achieved by using antimitotic compounds to double the ploidy level of haploid plants.

The haploid inducer (HI)-mediated genome-editing system is a promising approach for DH production that is still under development for the majority of crops. Nowadays, genome-editing technologies are of major importance in many research areas, plant breeding included. Since the discovery of CRISPR/Cas9 in 2012, and its first use in plants in 2013 (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013), many applications of genome-editing technology have been boosted thanks to the efficiency and versatility of CRISPR/Cas9 in comparison to

previous genome-editing technologies such as targeted induced local lesions in the genome (TILLING), zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). The CRISPR/Cas9 system is a genome-editing technology that enables an unprecedented control over the mutation process. CRISPR/Cas9 technology consists of a Cas9 nuclease, guided by a 20-nt gRNA sequence (gRNA), which induces DNA double-strand breaks (DSBs). DSBs are repaired by either non-homologous end-joining (NHEJ) or homology directed repair (HDR), generating insertion and deletion events (INDELs) in a precise DNA target sequence. CRISPR/Cas9 has features over its predecessors that make its use and application easier and more efficient (Belhaj et al., 2015).

Many reviews have been published summarizing haploid production and the best methodologies for each species. Many crops have excellent specific reviews focused on the DH techniques for a plant family or single species like the Fabaceae (Croser et al., 2007), the Solanaceae (Seguí-Simarro et al., 2011), the Cucurbitaceae (Dong et al., 2016), bell pepper (Irikova et al., 2011), wheat (Niu et al., 2014), rice (Mishra and Rao 2016), and sorghum (Teingtham and La Borde 2017). In addition, the use of antimitotic agents for plant tissue polyploidization has been reviewed by Dhooghe et al. (2011), and the current and novel technologies for haploid induction has been reviewed by Ren et al. (2017). However, an evaluation is still needed of the best methodologies for DH production that combine HI technology with the application of antimitotic agents for chromosome doubling in major crops. Moreover, the opportunities that HI-mediated genome-editing offers have not yet been reviewed for recalcitrant crop species.

In this review we discuss the following aspects in major crop species: the methods of DH production; the availability of chromosome-doubling methods to obtain DH lines; the opportunities for HI-mediated genome-editing systems in DH technology, and, finally, we focus on the development of the DH technique, HIs, antimitotic agents and chromosome-doubling protocols for the future of plant breeding.

DOUBLED HAPLOID USES

DH lines are highly important for plant breeding due to their complete homozygosis, making qualitative and quantitative phenotypic selection more efficient. Since the very first attainment of DHs in Brassica napus (Thompson 1972), many publications have reported the development of DH lines in more than 250 species (Maluszynski 2003). Following the research conducted in the 70s and 80s that demonstrated the ability to generate DHs in many cereal, vegetable and horticultural crops, the focus in recent decades has been optimizing and assaying different ways to enhance DH production in each species and genotype by introducing changes in every step of the DH programs.

DHs have been of great importance for: establishing chromosome maps and whole genome sequencing in the vast majority of genetically mapped and sequenced species; bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations; and, for mapping quantitative trait loci (QTLs) (Forster et al., 2007). This usage of DHs in basic research has been extended into direct application in variety improvements. Furthermore, DHs can be used as commercial cultivars such as stabilized homozygous lines or as parental lines to produce F1 hybrid lines, avoiding classical breeding methods to obtain stabilized and non-segregant lines.

STRATEGIES FOR THE PRODUCTION OF DOUBLED HAPLOID MATERIAL

Gametic haploid cells are the initial material used to obtain DH lines. Gametes from meiotic cells allow the generation of plantlets when cultured *in vitro* or when pollinated with irradiated pollen. The haploid step can be either a microspore from an anther or an ovule from an ovary depending on the species. The usual methods to induce haploids are as follows: androgenesis, gynogenesis and parthenogenesis (**FIGURE 3.3.1**). Plantlet regeneration from microspores or ovaries is a two-step protocol if a callus step is required prior to plantlet regeneration, or a one-step protocol if it directly induces an embryo or regenerates a plantlet. Besides, gametic cells from meiosis can be developed into haploid embryos, via parthenogenesis. Thus, a DH process always requires a gametic haploid step from which haploid or DH plantlet will be regenerated.

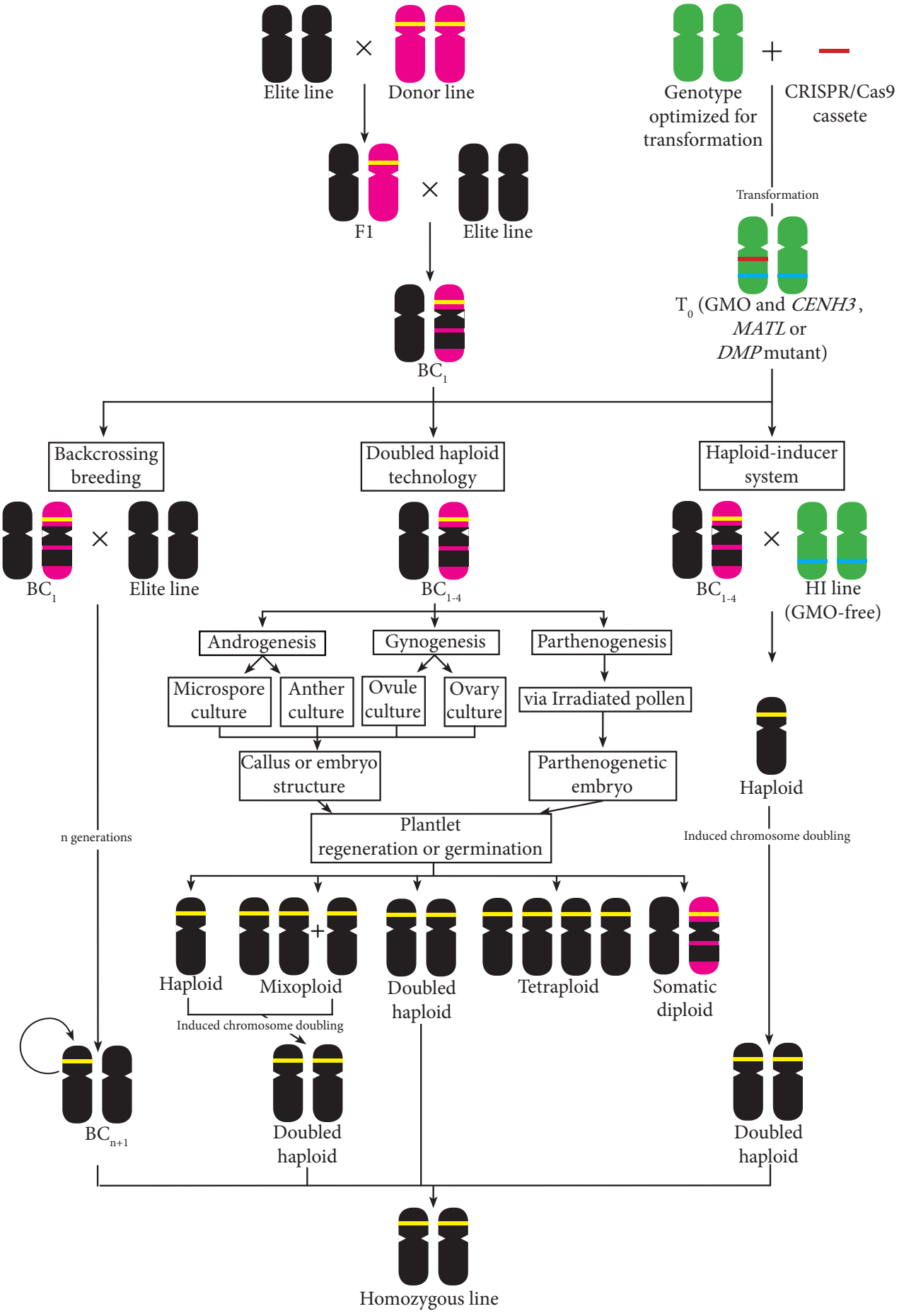
Elite line
Donor line
F1
Elite line
BC1
Genotype optimized for transformation
CRISPR/Cas9 cassete
Transformation
T0 (GMO and CENH3, MATL or DMP mutant)
Backcrossing breeding
Doubled haploid technology
Haploid-inducer system
BC1
Elite line
BC1-4
BC1-4
HI line (GMO-free)
Androgenesis
Gynogenesis
Parthenogenesis
Microspore culture
Anther culture
Ovule culture
Ovary culture
via Irradiated pollen
Callus or embryo structure
Parthenogenetic embryo
Haploid
n generations
Plantlet regeneration or germination
Induced chromosome doubling
Haploid
Mixoploid
Doubled haploid
Tetraploid
Somatic diploid
Induced chromosome doubling
BCn+1
Doubled haploid
Doubled haploid
Homozygous line

FIGURE 3.3.1. Schematic representation of three possible methods for pro- ducing purely homozygous lines: backcrossing breeding, doubled haploid technology and the HI-mediated CRISPR/Cas9 genome- editing system. Chromosome representation shows the genetic ploidy level according to number (one chromosome for haploids and two chromosomes for diploids) and the genetic background according to the color (black for elite receptor lines, pink for donor lines, green for genotypes optimized by genome transformation, yellow for the allele of interest, red for the CRISPR/Cas9 cassette targeting the *CENH3*, *MATL* or *DMP* gene and blue for the mutated *CENH3*, *MATL* or *DMP* gene). Backcrossing breeding can take from six to eight gen- erations depending on the species and possible coupled to marker- assisted selection. DH technology can take from 6 months to 2 years depending on the species. An HI-mediated genome-editing system can take 1 year. DH technology and HI-mediated genome-editing sys- tem can start with BC1 to BC4 plants.

In vitro culture techniques for gametic cells in androgenesis and gynogenesis techniques allow the original gametophytic pathway of the gamete be redirected towards a sporophytic pathway where plantlets can be regenerated. Androgenesis is the most common method to produce DHs (**TABLE 3.3.1**). Isolated microspores or microspores contained in anthers are cultured in specific induction media to induce the formation of callus. Subsequently, these calli are cultured in regeneration media to regenerate fully formed plantlets. In most cereal species, androgenesis is the only or the best method for DH generation with a high rate of spontaneous doubling, in species such as: rice (Hooghvorst et al., 2018), oat (Kiviharju et al., 2017) and bell pepper (Keleş et al., 2015). Gynogenesis stimulates *in vitro* embryogenesis development of the unfertilized haploid egg cells. In this process, a two-step protocol is usually carried out to induce callus formation from the female ovules in induction medium and to regenerate plants from callus in regeneration medium. For onion (Fayos et al., 2015) or beet (Hansen et al., 1995), gynogenesis is the best method for DH production. The ploidy level of the androgenetic and gynogenetic regenerated plants can differ depending on the cell events related to spontaneous or induced chromosome doubling (see below). Haploids, doubled haploids, mixoploids and tetraploids can be produced during the *in vitro* DH process (**FIGURE 3.3.1**). In androgenesis and gynogenesis it is desirable that the regenerated plantlets originate from microspore or ovule cells; nonetheless, somatic embryogenesis from anther or ovary tissues can take place. This process is defined as the regeneration of a whole plant from undifferentiated somatic cells in *in vitro* culture. The ploidy of these plantlets is diploid, and the genomic background is identical from which DHs are expected to be generated (**FIGURE 3.3.1**). Parthenogenesis methodology allows the formation of an embryo from an egg cell without fertilization. Egg cells can be induced to develop into haploid embryos following *in situ*

pollination with irradiated pollen, and these embryos only inherit the maternal set of chromosomes due to false fertilization. Such embryos germinate *in vitro* and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants. In the Cucurbitaceae, parthenogenesis is the only successful approach to obtain DH plant material (Dong et al., 2016).

Conventional DH technology has had great importance in some species to produce pure homozygous lines. Nonetheless, in all reported species there is a high genotypic dependency on the efficiency of the method, with some cultivars adapted to the existing protocols and some others very recalcitrant to the process. Publications on rice, wheat and maize show correlation with the substantial progress in haploid technology, attainable given the intensive research efforts (Croser et al., 2007). The majority of crops have acceptable DH protocols from which DH lines are produced successfully, but they require a significant time investment that ranges from five months to two years, substantial personnel and equipment needs, and always have inevitable variability in efficiency, depending on the genotype used. There are even some crops of great economic importance, including tomato species (Seguí-Simarro et al., 2011) and members of the Cucurbitaceae and Fabaceae family (Croser et al., 2007; Dong et al., 2016), that lack a successful beginning to end protocol for DH production.

TABLE 3.3.1. Overview of the most commonly used methods for doubled haploid production and chromosome doubling and their efficiency in major crops.

Species	Common name	Doubled haploid method	Induced chromosome doubling method					Chromosome doubling efficiency[b]		Reference
			Stage	Application	Antimitotic compound	Concentration	Exposure time[a]	Spontaneous	Induced	
Cereal crops										
Avena sativa	Oat	Anther culture	*In vitro* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	37.42%	88.17%	Kiviharju et al. 2017
Hordeum vulgare	Barley	Microspore culture	-	-	-	-	-	>90%	88.3-93.5%	Li and Devaux, 2003
Hordeum vulgare	Barley	Anther culture	*In vivo* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	-	-	Jacquard et al. 2003
Sorghum bicolor	Sorghum	Anther culture	-	-	-	-	-	95.3%	-	Kumaravadivel and Sree, 1994
Secale cereale	Rye	Anther culture	-	-	-	-	-	13-67%	-	Tenhola-Roininen et al. 2005
Triticum aestivum	Wheat	Microspore culture	Tillers of *in vivo* plants	Immersion	Colchicine	0.10%	5-8 h	-	95.60%	Niu et al. 2014
Triticum aestivum	Wheat	Microspore culture	Internode of pollinated spikes	Injection	Colchicine	1% suppl. 100 ppm 2,4-D	48 and 72 h	0	33-100%	Sood et al. 2003
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	250 mg·L^{-1}	48 h	31%	65.50%	Alemanno and Guiderdoni, 1994
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	300 mg·L^{-1}	48 h	0-0.18 DH green plantlets per 100 anthers	0.75 DH green plantlet per 100 anthers	Hooghvorst et al. 2018
Oryza sativa	Rice	Anther culture	*In vitro* plantlets	Immersion	Colchicine	500 mg·L^{-1}	5 h	-	35%	Hooghvorst et al. 2020a
Triticum x *Rye*	Triticale	Microspore culture	Embryos	Microspore culture	Colchicine	0.3 mM	24 h	30%	50-55%	Würschum et al. 2012
Zea mays	Maize	Anther culture	Anthers	Induction medium	Colchicine	0.2 g·L^{-1}	3 d	19%	20%	Obert and Barnabás, 2004

Zea mays	Maize	Anther culture	Microspores	Induction medium	Colchicine	1,250 µM	1 w	40%	93.75% DH plantlets for 100 anthers	Antoine-Michard and Beckert, 1997
Vegetable and horticultural crops										
Apiaceae or Umbelliferae										
Daucus carot	Carrot	Microspore culture	*In vivo* plants	Immersion	Colchicine	0.34%	1.5 h	50%	-	Ferrie et al. 2011
Pastinaca sativa	Parsnip	Microspore culture	*In vivo* plants	Immersion	Colchicine	0.34%	1.5 h	50%	-	Ferrie et al. 2011
Brassicaceae										
Brassica napus	Rapeseed	Microspore culture	Microspores	Induction medium	Colchicine	500 mg·L^{-1}	15 h	45-64.3%	83-91%	Zhou et al. 2002
Brassica oleracea var. *capitata*	Cabbage	Microspore culture	Rooted *in vitro* plantlets	Immersion	Colchicine	0.2% suppl. 2% DMSO	9-12 h	0-76.9%	58.3-75%	Yuan et al. 2015
Brassica oleracea var. *italica*	Broccoli	Microspore culture	Rooted *in vitro* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	6-12 h	50.6-100%	54.5-58.3%	Yuan et al. 2015
Cucurbitaceae										
Cucumis melo	Melon	Parthenogenesis	*In vivo* plants	Immersion	Colchicine	0.50%	2 h	-	46.03%	Solmaz et al. 2011
Cucumis melo	Melon	Parthenogenesis	*In vivo* plants	Immersion	Colchicine	0.50%	2 h	23%	20.93% DH and 9.30% mixoploids	Hooghvorst et al. 2020b
Cucumis sativa	Cucumber	Parthenogenesis	*In vitro* microcuttings	Culture medium	Colchicine	500 mg·L^{-1}	48 h	0%	30% DH and 55% mixoploid	Claveria et al. 2005
Cucumis sativa	Cucumber	Parthenogenesis	*In vitro* nodal explants	Immersion	Oryzalin	50 mg·L^{-1}	18 h	-	86.21%	Ebrahimzadeh et al. 2018
Solanaceae										
Capsicum annuum	Bell pepper	Anther culture	*In vitro* axillary buds	Lanolin paste in axillary buds	Colchicine	1%	-		n/a	Gyulai et al. 2000
Capsicum annuum	Bell pepper	Anther culture	-	-	-	-	-	22.2-53.4%	-	Keleş et al. 2015
Solanum melongena	Eggplant	Anther culture	*In vitro* axillary buds	Lanolin paste in axillary buds	Colchicine	0.50%	-	60%	25%	Corral-Martínez and Seguí-Simarro, 2012

Solanum tuberosum	Potato	-	Nodal segment	Immersion	Oryzalin	25 μM	8 h	20-78%	10.10%	Greplová et al. 2009
Other important plant crops										
Allium cepa	Onion	Gynogenesis	Embryos	Solid elongation medium	APM	25 μM	24 h	-	35%	Fayos et al. 2015
Allium cepa	Onion	Gynogenesis	*In vitro* plantlets	Culture medium	Colchicine	10 mg·L^{-1}	3 d	1%	46%	Campion et al. 1995
Allium cepa	Onion	Gynogenesis	*in vitro plantlets*	Media culture	Oryzalin	10 μM	3 d	-	67%	Jakše and Bohanec, 2003
Asparagus officinalis	Asparagus	Anther culture	*In vitro* shoot tips	Apical lanolin application	Colchicine	1.20%	-		21-97%	Tsay, 1997
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	Colchicine	0.40%	2.5 h	7.10%	4 DH per 100 ovules	Hansen et al. 1995
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	AMP	100 μM suppl. 1.5% DMSO	5 h	6.60%	4.7 DH per 100 ovules	Hansen and Andersen, 1998
Beta vulgaris	Beet	Ovule culture	*In vitro* plantlets	Agarose culture medium	Trifluralin	3.4 mg·L^{-1}	36 h	0-10%	62.50%	Gürel et al. 2000
Nicotiana tabacum	Tobacco	Anther culture	*In vivo* plant	Root dipping	Colchicine	0.50%	24 h		21-32%	Sood et al. 2013

[a] *h* hours, *d* days and *w* weeks

[b] If not specified, percentages refer to chromosome doubled material relative to the initially treated material

APPROACHES AND PROCESS OF CHROMOSOME-DOUBLING STEP

Every DH program starts with the haploid gametophytic phase to efficiently obtain DH plants. During the latter part of the *in vitro* process, haploid plant material needs to undergo chromosome duplication to finally obtain a fertile plant, from which DH seeds are recovered. The original chromosome set, whether maternal (gynogenesis and parthenogenesis) or paternal (androgenesis), must go through a spontaneous or induced duplication. The effective duplication of the haploid material is essential for the success of the DH process, because haploid plants are infertile. The chromosome doubling can be spontaneous or induced. Earlier duplication is the ideal for avoiding mixoploid plants, or fully haploid plants, and to ensure a battery of DH plants.

Endomitosis and nuclear fusion are the main causes of spontaneous duplication. These processes have been extensively studied in barley and inferred in other species (Kasha, 2005). During mitosis, chromosome multiplication and separation of cells usually occurs. Instead, in endomitosis, multiplication occurs but the cell fails to divide and one nucleus with two sets of chromosomes is restored. During nuclear fusion, two or more synchronized nuclei divide and develop a common spindle. Spontaneous chromosome doubling capacity during the process depends on the species and genotype. For example, the frequency of spontaneous DH androgenic bell pepper plants is 30-55% (Irikova et al., 2011; Keleş et al., 2015); in rice, it ranges between 8 to 30% of the regenerated plants (Alemanno and Guiderdoni 1994; Hooghvorst et al., 2018; López-Cristoffanini et al., 2018). In species whose spontaneous doubling rate is high, the process of induced chromosome doubling has not been explored to any great extent for obvious reasons. Nevertheless, despite this some genotypes are more likely to regenerate spontaneous DHs than others because all *in vitro* processes are genotypically dependent, including duplication. Indeed, species with generally high rates of spontaneous DH still need efficient protocols to induce chromosome doubling with antimitotic compounds because some genotypes within such species have low rates of spontaneous DH generation or no spontaneous production at all, meaning that antimitotic application is still essential.

A proportion of the so-called 'spontaneous' duplication reported in the literature is actually induced via chromosome doubling by means of pre-treatments like heat or cold pre-treatments that do not involve antimitotic.

Temperature stress, like heat or cold pre-treatments, is usually applied during androgenesis and gynogenesis prior to *in vitro* culture. Many of these pre-treatments were originally intended to increase microspore induction, but they usually increase the frequency of chromosome doubling due to the destabilization of microtubules and microfilament elements that form the cytoskeleton (Kasha, 2005). In microspores, cold pre-treatment is related to failure of cell wall formation leading to multinucleate structures (known as coenocytic structures, see Testillano et al. (2002)) which result from nuclear division without cytokinesis. However, in spite of the basic research that has related pre-treatment protocols to increased number of microspores at the optimal stage, once the microspore culture is started and plants regenerate, it is difficult to demonstrate that the pre-treatment is the causal agent of the increase in frequency of DHs beyond the determination of the best microspore stage for embryogenesis.

Induced chromosome duplication may be feasible at different stages of the process: at the first pollen mitotic division of microspore cells, at the callus stage when growing *in vitro*, or at the plant stage when regenerated (**TABLE 3.3.1**). The use of antimitotic compounds is mandatory when spontaneous chromosome doubling is absent or very low. A specific type of endomitosis, known as C-mitosis, takes place when antimitotic compounds are used to destabilize the cell cycle, perturbing not only mitosis but also arresting cells during interphase (Lu et al., 2012). In interphase, DNA is replicated and each replicated chromosome forms sister chromatids that are bound by the centromere's spindle tubules. When C-mitosis occurs, the antimitotic compound interacts with tubulin subunits destabilizing and inhibiting their assembly. Antimitotic treatment depends on the species and the protocol used for obtaining DH plants. Some key features considered are the antimitotic agent, its concentration, the exposure time and the treatment stage, which were thoroughly reviewed by Dhooghe et al. (2009).

In androgenetic protocols for DH production, free-microspores or microspores contained in anthers are usually cultured in colchicine-supplemented medium. By this means, chromosome doubling is achieved at earlier stage ensuring the success of the process. In addition, an increased level of microspore induction has been described attributed to the presence of colchicine (Alemanno and Guiderdoni, 1994; Iqbal et al., 1994; Barnabás et al., 1999; Hooghvorst et al., 2018).

Haploid plants as explant material are another important source for recovering DHs. Most of the DH protocols apply antimitotic compounds to plants grown either *in vitro* or *in vivo* to achieve chromosome doubling

(**TABLE 3.3.1**). Immersion of the whole *in vitro* plantlet or the apical meristem *in vivo* are two approaches that usually yield good chromosome doubling results in many plant species (**TABLE 3.3.1**). *In vitro* treatments usually take longer and the antimitotic concentration is lower, whereas *in vivo* treatments have shorter exposure times with higher concentrations of compound. Nevertheless, in some species like onion, the apical meristem of adult plants is inaccessible, impeding chromosome doubling *in vivo* (Bohanec 2002). Meanwhile, during propagation of regenerated haploid plants chromosome doubling has also been achieved by adding antimitotic compounds to the culture medium. Breeders sometimes discard androgenetic and gynogenetic haploid plants when spontaneous doubled regenerants are considered acceptable. Despite their great potential, the lack of a successful protocol for chromosome doubling in grown plants means that they frequently go to waste. In contrast, the parthenogenetic process usually depends on whole plants or embryos as the source of material for DH (**TABLE 3.3.1**).

Several antimitotic agents have been used for chromosome doubling of haploid plants. Colchicine is the most popular antimitotic agent used for DH studies in most species because it has a high chromosome doubling ability. Yet there are many different chemicals with antimitotic effects such as amiprophosmethyl (AMP), pronamide, propham, oryzalin and trifluralin, which have similar effect and mechanisms of action to colchicine (Bartels and Hilton 1973; Lu et al., 2012). Colchicine is a toxic natural product extracted from plants of the Colchicum genus and used as an antimitotic agent. It is known to inhibit mitosis in a wide variety of plant and animal cells by interfering with the structure of the mitotic spindle (Eigsti and Dustin 1955). Furthermore, research shows that the colchicine-binding protein is a subunit of microtubules. It has been reported that low dosages of the compound can effectively halt cell division for a small period of time thus producing a doubling of the genetic load in some cells (Borisy and Taylor 1967). Nevertheless, colchicine has a highly toxic effect on plant and animal cells, being a hazardous compound for researchers to use in the laboratory. Moreover, when plants are treated with colchicine to induce doubling, the mortality rate is usually high, and is dependent on the concentration, time of exposure and species. Consequently, to optimize protocols, breeders need to balance dose and exposure time to ensure successful chromosome duplication while limiting the mortality rate.

Nowadays, oryzalin and trifluralin are being widely employed as substitute for colchicine. These compounds are dinitroaniline herbicides reported to bind to plant tubulin, which in turn confers an antimitotic effect similar to colchicine. Unlike colchicine, dinitroanilines have no effect on microtubules in vertebrate cells,

which are resistant to its depolymerizing effects. It has been demonstrated that oryzalin has a high-affinity interaction with plant tubulin, binding rapidly and reversibly while forming a tubulin-oryzalin complex (Hugdahl and Morejohn 1993). The properties of dinitroaniline binding to tubulin are different from colchicine. In fact, dinitroaniline binding is time independent and the tubulin-dinitroaniline complex dissociates completely, unlike colchicine, which has been reported to bind slowly to tubulin and the tubulin-colchicine complex does not easily dissociate. Furthermore, oryzalin has been reported to have a much higher affinity for unpolymerized tubulin than the polymerized form (Borisy and Taylor 1967; Hugdahl and Morejohn 1993). Scientific interest lies in the fact that dinitroanilines can be much less hazardous for humans than colchicine and equally effective at lower doses.

Key features commented above for a successful chromosome doubling need to be determined empirically for each species. More work is required concerning the chromosome-doubling step in many species and genotypes, describing the best results of different treatments (**TABLE 3.3.1**).

The antimitotic compound is usually dissolved in dimethyl sulfoxide (DMSO). DMSO has a double utility, as a solvent to dissolve the antimitotic and to increase cell permeability by allowing an increase in absorption of the agent into the plant (Hamill et al., 1992). However, DMSO may increase plant mortality due to its relative toxicity (Dhooghe et al., 2011). Other solvents can be used instead, such as NaOH or 70% ethanol for oryzalin, acetone for trifluralin, or even water (Dhooghe et al., 2011). If chromosome doubling is performed by immersion of some part of the plant in an antimitotic solution, detergent or surfactants can be added too to enhance the surface contact. In contrast, if the treatment is applied to a specific area on the plant, such as the lateral or apical meristems, lanolin paste can be used to localize a dose of solution.

CRISPR/Cas9: A NEW ACTOR IN DH TECHNOLOGY

The haploid induction strategy is based on intraspecific crossing to obtain haploid progeny through an HI line. HI lines have the ability to produce haploid embryos upon pollination of a receptor line. Due to a mutation in a specific gene, which is essential for the normal fertilization of female gametic cells, fertilization is impeded, and egg cells develop into haploid embryos. Natural HI lines have been used since the beginning of modern breeding in maize (Coe 1959), barley (Kasha and Kao, 1970), tobacco (Burk et al., 1979) and wheat (Laurie and Bennett 1988). For instance, in maize, HI lines were discovered to carry a 4-bp insertion in the carboxy terminus of the *MATRILINEAL* (*MATL*) gene, also known as *NOT LIKE DAD* (*NLD*) (Gilles et al., 2017) or *PHOSPHOLIPASE A1* (*PLA1*) (Liu et al., 2017), which encodes a pollen-specific phospholipase determined as the causal factor in the haploid induction process (Prigge et al., 2012). Phospholipase are essential enzymes expressed during pollen development and play a critical role in pollen development and germination and tube growth, its mutations are associated with delayed pollen germination and tube growth (Kim et al., 2011). Nevertheless, few species have natural HI lines; the production of HI lines in the laboratory through genome-editing techniques is a major challenge in haploid technology to improve the DH process. As previously mentioned, TILLING, ZFNs and TALENs are potential genome-editing tools to produce positive mutants. Despite this, the random mutations of TILLING, and the complex, time-consuming engineering and unwanted off-target mutations of ZFNs and TALENs have meant that the CRISPR/Cas9 system has become genome-editing system of choice.

DH technology has experimented a substantial progress resulting from the advances in engineered HI lines. Recently, *MATL* has become a target gene for genome editing in rice and maize with the CRISPR/Cas9 system, taking advantage of gene mutation during haploid induction (**TABLE 3.3.2**). In maize, maternal haploids were obtained with an efficiency of ca. 6.7% using the HI technique (Kelliher et al., 2017) and in rice, the average haploid induction rate was ca. ~6% (Yao et al., 2018). In wheat, a difficult species to work with because of their polyploidy, a 18.9% of haploid progeny was obtained using *MATL*-edited plants (Liu et al., 2019). On the other hand, another promising gene for HI, *DMP* (DOMAIN OF UNKNOWN FUNCTION 679 membrane protein), was demonstrated to induce haploids with a rate of 0.1%-0.3% in maize. *DMP* is a pollen-expressed gene highly

expressed during the late stage of pollen development and localized to the plasma membrane (Zhong et al., 2019). Despite of the lower rate of HI due to the loss-of-function of *DMP* gene, the combination of *ZmDMP* and *ZmPLA1* (or so-called *MATL* or *NLD*) genes knockout in the *CAU6*$^{(zmpla1\text{-}zmdmp)}$ line triggered and enhanced HI rate by 5 to 6-fold (Zhong et al., 2019) (**TABLE 3.3.2**). These rates of haploid production represent a breakthrough point that has the potential for further improvements. This new DH-generation pathway could be implemented for other secondary cereals like barley, oat, rye or triticale because existing DH protocols are less efficient than in species like rice, maize or wheat. With many genetic transformation methods validated and whole genome sequencing available, breeders should be able to take advantage of haploid induction validated technology to increase the number of DHs and the efficiency of DH production in recalcitrant species via HI-mediated genome-editing technology, avoiding the need to test *in vitro* androgenic protocols for a range of genotypes.

TABLE 3.3.2. Summary of the haploid inducer-mediated genome-editing systems using CRISPR/Cas9.

Species	Common name	Target gene	Haploid induction rate	Reference
Monocotyledonous				
Triticum aestivum	Wheat	*MATL*	18.9%	Liu et al. 2019
Oryza sativa	Rice	*MATL*	2-6%	Yao et al. 2018
Zea mays	Maize	*DMP*	0.1-0.3%	Zhong et al. 2019
Dicotyledonous				
Arabidopsis thaliana	Arabidopsis	*DMP*	2.1%	Zhong et al. 2020
Arabidopsis thaliana	Arabidopsis	*CENH3*	1.1-44%	Kuppu et al. 2020

Concerning dicotyledons, the main target for the HI technique has been the *CENH3* (centromeric histone 3) gene, which is a histone present in all plants that determines the position of the centromere and like other histones, it carries an N-terminal tail, which protrudes from the nucleosome and is a target for posttranslational modification and a C-terminal Histone Fold Domain, which interacts with DNA and other histones to form the nucleosome. *CENH3* plays a major role in chromosome segregation during mitosis, its alteration may have severe or even lethal consequences (Britt and Kuppu 2016). Since its discovery, much research has been conducted to elucidate its function and capacity during haploid induction in dicot species. Nevertheless, the specific mechanism and functions are still not clear. In 2010, major progress was achieved when studying the function of I in Arabidopsis thaliana (Ravi and Chan 2010). This work demonstrated that GFP-tailswap (*cenh3-1* mutant plants)

plants underwent haploid induction when crossed with a *CENH3* wild type line by eliminating chromosomes of the mutant line. Recently, the late HI related gene discovered was *DMP* was used for haploid induction in dicots. The loss-of-function of the *DMP* gene in Arabidopsis, a *ZmDMP*-like gene, induced haploid progeny in a rate of 2.1% (Zhong et al., 2020) (**TABLE 3.3.2**). Besides, the *CENH3* gene was disrupted in maize too through *CENH3*-tailswap transgenic complementation, reaching a 3.6% of HI (Kelliher et al., 2016).

Hence, the *MATL*, *CENH3* and *DMP* genes are the current targets for HI-mediated genome-editing systems in monocots and dicots. However, other genes related to chromosome segregation during mitosis, or pollen development have potential as target genes for HI technology using CRISPR/Cas9, as reviewed by Ren et al. (2017).

Parthenogenetic approaches of haploid production via HI lines obtained by CRISPR/Cas9 can increase the chances of DH plant generation and ease the usual time-consuming and labor-intensive processes of androgenesis, gynogenesis and parthenogenesis (**FIGURE 3.3.1**). Further, the genotype dependency of many of the *in vitro* steps, like callus induction and plant regeneration, or *in vivo* steps like pollination with irradiated pollen, might be avoided using an HI approach, and this may also enable haploid plant material to be obtained from recalcitrant genotypes. To obtain *MATL*-, *CENH3*- or *DMP*-mutated lines for use as HIs, the transformation should be optimized in at least one genotype for each species because the regeneration and mutation processes are mandatory. This HI mutated genotype could be used to pollinate many genotypes of interest for haploid embryo generation, avoiding the need to optimize the process for recalcitrant genotypes.

The HI-mediated genome-editing approach for DH production for breeding purposes is a major discovery. Apart from improvements in the application of HIs discussed above, for a number of crop species the HI technique might be the best, if not the only way, to produce DHs. Much research needs to be done on this aspect to confirm the ease of work in parallel with better results. If the production of haploid plants is more efficient using the HI approach only the chromosome-doubling step at the haploid plant stage will become the bottleneck in achieving an efficient production of DH lines (**FIGURE 3.3.1**).

Another important approach to the HI technique is the HI-Edit system, where successful one-step genome-editing is achieved. Kelliher et al. (2019) crossed HI lines carrying the CRISPR/Cas9 cassette targeting

genes for phenotypical evaluation with inbred lines to test the ability to produce positive genome-edited mutants in the haploid offspring. The intraspecific crossing in maize led to a mutant haploid descendance of 2 to 8%, depending on the target gene. The interspecific crossing between a wheat inbred line and a maize line homozygous for the Cas9 gene, resulted in a 1.8% rate of mutant haploids. Moreover, in Arabidopsis, 17% of the offspring were mutant haploids when crossing a HI line with CRISPR/Cas9 system with an inbred line. Wang et al. (2019) applied the same system in maize and obtained 10 positive genome-edited plants among 245 haploids. This new system enables direct editing of elite inbred lines via a single crossing, thus overcoming recalcitrance.

CHALLENGING FAMILIES: SOLANACEAE; FABACEAE and CUCURBITACEAE

In plant breeding, important crops usually have a higher economical investment in order to increase their traits of interest. This is translated into a higher number of protocols and methodologies available for those species. DH technology follows the same pattern. However, sometimes, technical limitations related to a species or a genotype may rise difficulties to adapt a technique. It is the case of DH methodology in important families such as Solanaceae, Fabaceae and Cucurbitaceae, which despite of their economic worldwide importance, the progress achieved until nowadays is relatively low, in some cases.

It is worth noting that DH production in crop species like those from the Solanaceae could be greatly improved thanks to the HI technique. Solanaceae species are very recalcitrant to *in vitro* DH processes and a methodology has not been established yet for a number of species, making it difficult for breeders to use DH technology on a routine basis (Seguí-Simarro et al., 2011). For instance, DH lines can be obtained efficiently in eggplant and bell pepper through anther culture (**TABLE 3.3.1**). Nevertheless, to our knowledge there is currently no suitable DH method available for tomato, despite all the efforts invested in DH production in this major horticultural crop. Thus, classical breeding is the only method to obtain new commercial tomato cultivars, complicating the advances of breeding selection. Therefore, generation of a HI *CENH3*-mutant tomato lines via genome-editing could represent a breakthrough. This could lead to a new era for tomato breeding, avoid the current generations of self-pollination that are still required to produce inbred lines for use in hybridization. Fortunately, delivery of CRISPR/Cas9 by *Agrobacterium*-mediated transformation has been reported several times in tomato (Van Eck 2018). CRISPR/Cas9 has been successfully applied in tomato with mutation efficiencies of 80-100% for applications such as studying mutation stability of heredity in later generations (Pan et al., 2016), obtaining parthenocarpic tomato fruits (Ueta et al., 2017), and increasing plant resistance to powdery mildew (Nekrasov et al., 2017).

In the Fabaceae, attempts to produce DHs have been reported in many species (soybean, field pea, chickpea, peanut and common bean), mainly via androgenesis. Leguminous species are particularly important for low input and sustainable cropping due to their ability to fix nitrogen and as a dietary protein source for human food and animal feed (Croser et al., 2007). Nonetheless, not much progress has been made with DH technology

applications because there has been little research undertaken on these species, and the induction and regeneration rates are inherently low. Some DH lines have been produced thanks to spontaneous doubling and high rates of somatic regeneration (Croser et al., 2007; Ochatt et al., 2009). However, successful induction of chromosome doubling has not been widely reported in this family because of the scarcity of the obtained haploid material. Significantly, the CRISPR/Cas9 system has been applied in soybean multiple times (Chilcoat et al., 2017; Sun et al., 2015) and should be extended to other Fabaceae species. The HI-mediated CRISPR/Cas9 genome-editing technique presents a great opportunity to produce DHs in these species.

Another important family where HI-mediated genome-editing system could be advantageous is the Cucurbitaceae. Parthenogenesis via pollination with irradiated pollen is the best-known method to obtain haploid material among these species. However, the efficiency of the process is usually impeded by: (i) a high genotypic dependency; (ii) a low production of haploid embryos; and (iii) a difficulty to induce chromosome doubling of haploid plants due to mortality, hyperhidricity and a high ratio of haploids and mixoploid plants (Dong et al., 2016; Hooghvorst et al., 2020b). All this makes the process of DH production time-consuming and inefficient. The HI approach in the Cucurbitaceae would be similar to parthenogenesis via irradiated pollen due to the initial pollination of the receptor plant with haploid-inducer pollen, and the rest of the process would be the same. Additional research is necessary to confirm whether the use of an HI line results in increased production of haploid embryos, reported rates lie between 0 and 5% of seeds containing haploid embryos (Hooghvorst et al., 2020b). There are a few reports of the CRISPR/Cas9 genome-editing system being applied to cucumber, watermelon and melon with mutation efficiencies ranging from 42 to 100% (Chandrasekaran et al., 2016; Hu et al. 2017; Tian et al., 2017; Hooghvorst et al., 2019). On other important cucurbit species, such as *Cucurbita maxima*, *C. moschata* and *C. pepo*, there are no reports of successful generation of parthenogenetic DHs via irradiated pollen nor the application of CRISPR/Cas9 system. Nevertheless, HI-mediated genome-editing should afford great opportunities for breeding in these species as well.

Despite all the other improvements in DH line production that have emerged, chromosome doubling is one step that has been inherited from the classical DH approach (**FIGURE 3.3.1**). It is therefore of major importance to adapt and optimize new chromosome-doubling protocols via antimitotic compounds to increase the number of DH lines derived from the improved HI protocols. Some species, such as sorghum and bell pepper,

do not have an optimized well-described chromosome-doubling protocol because of their high level of spontaneous DH regenerants (**TABLE 3.3.1**). For instance, in rice, the spontaneous chromosome-doubling rate is usually very high, ranging from 30 to 80% (**TABLE 3.3.1**), and induced chromosome doubling has been ignored since the generation of the first DHs (Niizeki and Oono 1968). Indeed, before 2019 no reliable reports on doubling rice plants were published, authors treated androgenic haploid plants with a recovery of 35% (Hooghvorst et al., 2020a). On the other hand, species whose DH production is impeded by poor performance in tissue culture do not have a reliable described method for chromosome doubling; this is the case of rye, watermelon, other secondary cucurbit species, tomato and leguminous species (Croser et al., 2007; Forster et al., 2007; Seguí-Simarro et al., 2011; Dong et al., 2016).

HI-CRISPR/Cas9 LEGISLATIVE FUTURE

Another important aspect of HI-CRISPR/Cas9 based technology is the legislation put in place to regulate the development and commercialization of genetically modified organisms (GMOs) in the EU, and which handles issues of uncertainty and safety (Sprink et al., 2016). The Directive 2001/18/EC defines a GMO as an organism "in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination", and GM techniques are further described as "recombinant nucleic acid techniques involving the formation of new combinations of genetic material". In our opinion, haploid production through CRISPR/Cas9 mutants used for HI needs to be exempt from this regulation because the resultant GMO-free haploid line would never have recombinant DNA incorporated into its single progenitor cell.

The US regulatory framework would categorize HI-CRISPR/Cas9 as a null segregants because transgenic sequences are eliminated from the final product. However, this technique has the precedent of centromere-mediated chromosome elimination (CCE) through a GFP-tailswap transgenic line for which the Plant Health Inspection Service (APHIS) determined that the progeny obtained from CCE would not be subjected to its regulation (Camacho et al., 2014).

Nevertheless, as pointed out by Abbott (2015) and (Camacho et al., 2014), the EU and the US criterion in the current legislation is based on the process rather than the product. Therefore, a process that uses recombinant technology could create controversy for regulatory institutions, despite the impossibility of detecting the haploid origin. The regulatory framework should be based on science and able to evolve together with the scientific knowledge and technologies.

PLOIDY-LEVEL IDENTIFICATION OF PLANT MATERIAL

Ploidy identification of the plants produced during the DH process is essential before the chromosome-doubling treatment to determine whether the plant material has undergone spontaneous duplication, and afterwards to check whether or not the antimitotic treatment has successfully doubled the plants' chromosomes. There are multiple methods to check ploidy level: cytologically, morphologically, via marker-assisted selection or via flow cytometry method.

Cytological procedures for ploidy level determination can be carried out by counting chromosomes or examining the epidermal tissue of the leaves. Chromosome counting usually uses root tip cells, which are fixed, and the chromosomes are then stained and observed for counting (Maluszynska 2003). Cytological analysis of leaves correlates chloroplast number, stomata dimensions and size with ploidy level. However, applying chromosome-counting methods to species with small chromosome size is very time-consuming and difficult, and chloroplast and stomata analysis is species- and genotype-dependent. Despite this, results from both cytological procedures are extremely accurate and sample preparation and staining is easy and fast.

Haploid and diploid plants can also be distinguished according to morphological observations of the plant material. Morphological observation is based on comparing plant traits of the donor plants and regenerants, such as: height, vigor, leaf shape, flower development, fertility and presence of pollen. This methodology does not require special equipment, but it is sometimes unreliable and subject to environmental effects. In the Cucurbitaceae family, leaf morphology, flower shape and size, pollen production, stem length or node number, are phenotypic variations that can be analyzed for ploidy determination (Dong et al., 2016). Couto et al. (2013) correlated haploid levels with small plant size and brittle leaves, but excluded ploidy determination of haploid seeds via morphometric parameters due to the great variability in the seeds. Yuan et al. (2015) detected haploid individuals in two Brassica species due to the weak growth of the haploids and the small size of the plants as wells as the presence of smaller flower buds, the absence of pollen, and a lack of stamens in the flowers.

The use of segregating alleles in the donor parent is another methodology for ploidy level determination via marker-assisted selection. Simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) marker analysis techniques are ideal to identify ploidy-level

as well as homozygosity in spontaneous DH regenerants or heterozygosity in diploid somatic regenerants. For instance, in soybean, 114 androgenic embryo-like structures were analyzed for the Satt418 SSR marker, and 74% were found to be heterozygous originating from somatic anther tissue (Rodrigues et al., 2004). In pumpkin, 23 SSR markers were screened in 253 parthenogenetic diploid plants and the results showed no spontaneous chromosome doubling (Košmrlj et al., 2013). In oat, (Kiviharju et al., 2017) DNA markers were used for selection and this indicated that 3.4 % of regenerated androgenic plants were heterozygous.

Despite all the methods described above for ploidy-level determination, flow cytometry is the most used method for a wide range of species because of its convenience and rapidity estimating the nuclear genome size in plants (Doležel et al., 1998) . Estimation of ploidy-level via nuclear suspensions of plant cells and chromosome staining has been used in DH programs for routine laboratory analysis. Many optimizations have been done in order to increase the number of nuclei in suspension using extraction methods such as chopping and bead beating, alongside with numerous modifications of isolation buffers and staining procedures (Doležel and Barto, 2005; Hooghvorst et al., 2019). Moreover, in addition to haploid and diploid cells, flow cytometry is especially useful for detecting triploid, tetraploid and mixoploid plants. On the other hand, flow cytometry requires highly specialized equipment that is not required with earlier methods of ploidy determination.

CONCLUSIONS

The attempt of plant breeding and DHs to increase productivity and other important plant traits makes it necessary to continue refining existing and new methods to finally achieve a more sustainable production according to social needs. We have undertaken an analysis of DH production methods coupled with chromosome-doubling protocols for production of DH plants in major crops. We have drawn general conclusions about the success of different approaches to DH generation and the implications of the existing technologies of DH production and chromosome doubling for future research. DH technology has been a major boost for plant breeding, reducing the time and labor required to derive new breeding varieties. Among the DH processes, chromosome doubling is often overlooked due to the importance of the haploid induction step or the high frequency of spontaneous DHs, and this is reflected in the absence of DH protocols or a lack of efficiency in those that exist. Androgenesis via anther culture is the most common protocol for haploid and DH plant production, being the predominant method in vegetable and horticultural crops, and the only method used in cereals. The chromosome-doubling step is far from being settled and there is a need to continue investigating new protocols based on new or existing antimitotic compounds in order to reduce toxicity-related mortality and to attain higher frequencies of chromosome doubling. Induced chromosome doubling has a genotypic dependency, and even species with a high rate of spontaneous doubling should not be ignored when developing efficient chromosome-doubling methods because some genotypes are unable to regenerate spontaneously. Inducing haploids using genome editing via the CRISPR/Cas9 system could revolutionize the whole process of haploid generation and DH production, and should have an impact on plant breeding in the coming years to parallel the early days of the DH technique. For crops with a short progress on DH technology, CRISPR/Cas9 HI approach could open a new insight, allowing the production of pure homozygous lines. For many of the vegetable crops and secondary cereals, like tomato, Fabaceae, Cucurbitaceae, barley, oats, rye and triticale, HI systems will allow researchers to avoid the need for wide ranging adaptation of protocols to different genotypes, which is a highly time-consuming pathway requiring much trial and error. The only adaption required to the tissue culture technique will be the regeneration of CRISPR/Cas9 genome-edited plants, which will be a much easier task because only one genotype will be sufficient as the pollen donor for haploid induction across a diversity of receptor genotypes

of the same species. In the future, HI-mediated genome-editing CRISPR/Cas9 system should be exempted from GMO legislation due to the absence of GMO in the maternal antecessor and haploid descendancy. The uprising CRISPR/Cas9-based gene targeting approach for haploid induction will make the chromosome-doubling step inexorable, because of the low or absent spontaneous chromosome doubling in haploid induction process. Therefore, new *in vitro* or *in vivo* chromosome-doubling protocols will be needed for species where haploid induction has not yet been reported due to low regeneration efficiency or a complete lack of regeneration success.

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CHAPTER IV. Opportunities and Challenges in Doubled Haploids and Haploid Inducer-Mediated Genome-Editing Systems in Cucurbits

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ABSTRACT

Doubled haploids have played a major role in cucurbit breeding for the past four decades. *In situ* parthenogenesis via irradiated pollen is the preferred technique to obtain haploid plantlets whose chromosomes are then doubled in Cucurbitaceae, such as melon, cucumber, pumpkin, squash and winter squash. In contrast to doubled haploid procedures in other species, *in situ* parthenogenesis in cucurbits presents many limiting factors which impede efficient production of haploids. In addition, it is very time-consuming and labor-intensive. However, the haploid inducer-mediated genome-editing system is a breakthrough technology for producing doubled haploids. Several reports have described using the CRISPR/Cas9 system in cucurbit species, and although its application has many bottlenecks, the targeted knock-out of the *CENH3* gene will allow breeders to obtain haploid inducer lines that can be used to obtain parthenogenetic embryos. In this review, we discuss the progress made towards the development of doubled haploids and haploid inducer genotypes using CRISPR/Cas9 technologies in cucurbit species. The present review provides insights for the application of haploid inducer-mediated genome-editing system in cucurbit species

INTRODUCTION

The Cucurbitaceae is a family with several important crop species and contains a great genetic, morphologic and phenotypic variability. In terms of worldwide production, the most important species are watermelon with 104 m tons, cucumber and gherkins with 75 m tons, pumpkin, squash and gourds with 28 m tons and melon with 24 m tons (FAO, 2018). Commercial cultivars are usually F1 hybrids due to heterosis, which results in earlier harvest, increased yield and higher vigor (Robinson, 2000).

Therefore, pure lines used as parents for hybrid production are invaluable. There are two main methods in cucurbits to produce completely homozygous lines, classical breeding and doubled haploids. Classic breeding requires several rounds of selfing and selection for eight to ten generations, which consumes a considerable amount of time and resources. On the other hand, doubled haploid (DH) methodology allows the efficient production of completely homozygous pure lines in less than two years. Parthenogenic DH methodology in cucurbits consists of two basic steps, the initial production of haploid material through *in situ* parthenogenesis via pollination with irradiated pollen and the subsequent chromosome doubling of haploid plants to restore the diploid chromosome content and to allow the generation of DH seed. Nevertheless, parthenogenesis in cucurbits has several bottlenecks and limitations that may jeopardize the final production of DH lines, such as high genotypic dependency; low fruit set when pollinated with irradiated pollen; difficulties in detecting parthenogenetic embryos; low production of parthenogenetic embryos; recalcitrant culture *in vitro*; high mortality of parthenogenetic plants during *in vitro* culture and after the chromosome doubling treatment; low rates of chromosome doubling; and low fruit set of DH lines once chromosomes are doubled (Hooghvorst et al., 2020).

DHs have been widely used to obtain stabilized homozygous lines to be used as parental lines to produce F1 hybrids. On the other hand, stabilized lines have been used for: establishing chromosome maps and whole genome; bulked segregant analysis (BSA); and, for mapping quantitative trait loci (QTLs) (Forster et al., 2007).

Until recently, classical breeding and DHs were the main pathways for the production of parental lines. However, the haploid inducer-mediated genome-editing system is a breakthrough approach that could lead to a new era of cucurbit breeding. The currently-predominant genome editing technique is CRISPR/Cas9 due to its efficiency, versatility, and unprecedented control over mutation. CRISPR/Cas9 consists of a Cas9 nuclease guided

by a 20-nt sequence (gRNA). This system induces DNA double-strand breaks (DSBs) that are repaired by either non-homologous end-joining (NHEJ) or homology directed repair (HDR), generating insertion and deletion events (INDELs) (Bortesi and Fisher, 2015).

There are numerous reports and reviews of DH production and CRISPR/Cas9 applications in many plant species including cucurbits. Nevertheless, haploid inducer-mediated genome-editing systems have not yet been applied in cucurbits. Therefore, the purpose of this review is to focus on haploid inducer-mediated genome-editing systems in cucurbit species to provide new insights, opportunities and challenges, which may be valuable for developing this technique in cucurbits and other species.

DOUBLED HAPLOIDS PROCEDURE IN CUCURBITS

Doubled haploid lines in cucurbit species can be produced by parthenogenesis, androgenesis or gynogenesis. However, parthenogenesis is the currently-predominant technique to produce doubled haploids in cucumber, melon, watermelon and pumpkins (Dong et al., 2016). The parthenogenesis process starts with *in vivo* pollination using irradiated pollen. The resulting parthenogenetic embryos are then detected, rescued and cultured *in vitro*, in order to germinate and develop into plantlets. The ploidy-level of parthenogenetic plantlets is estimated by flow cytometry and can result in haploid, spontaneous doubled haploid, mixoploid or tetraploid levels. The haploid and mixoploid plantlets need to undergo *in vitro* or *in vivo* chromosome doubling, usually using colchicine as antimitotic compound. Once the plantlets have doubled their chromosomes, they are cultured in the greenhouse together with the spontaneously-doubled haploid lines to recover DH seed (Hooghvorst et al., 2020; Dong et al., 2016; Lim and Earle, 2008). During the DH process, a high genotypic dependency and other factors continuously hamper each step causing a loss of efficiency that might be critical.

Pollination with Irradiated Pollen and Fruit Set

The first step of the *in situ* parthenogenetic process is the irradiation of mature pollen and the pollination of the receptor female flowers. γ-ray (60Co or 137Cs) and soft X-ray are the usual irradiation sources applied to male flowers. The ionized pollen can germinate on the female stigma and grow pollen tubes to reach the embryo sac. However, this pollen is genetically inactive and unable to fertilize the egg-cell and the polar nuclei. Therefore,

irradiated pollen stimulates egg-cell division and parthenogenetic embryo induction (Hooghvorst et al., 2020). Overall, the dose of ionizing radiation can range from 25 to 500 Gy, depending on the species and can yield less parthenogenetic haploid embryos at higher or more diploid embryos at lower dosages. Therefore, irradiation should be optimized for each species because pollen sensitivity is attributed to radiation-resistance.

The parameters that define the success or failure of pollination with irradiated pollen are the number of fruits set and the ratio of parthenogenetic embryos per fruit. Fruit set is lower when pollination is performed using irradiated pollen (Hooghvorst et al., 2020). The number of female flowers that develop into a fruit can range between 10–25% in melon (Hooghvorst et al., 2020), 20–25% in pumpkin (Košmrlj et al., 2013), or 50% in cucumber (Smiech et al., 2008). The initial number of parthenogenetic embryos is crucial to have enough plant material during the whole process. The plant material usually decreases progressively during each step of the DH process, due to mortality, inefficiency of the method and recalcitrant performance. Frequently, the ratio of parthenogenetic embryos per fruit is low, 0.23–5.79 in cucumber (Smiech et al., 2008; Claveria et al., 2005); 0.2–16 in pumpkin, squash and winter squash (Košmrlj et al., 2013, Kurtar et al., 2002, 2009, 2010; Baktemur et al., 2014); 0.3–6 in melon (Dong et al., 2016; Hooghvorst et al., 2020); or 1.4 in watermelon (Taškın et al., 2013).

In addition, the genotype of the donor and the receptor plants have an influence in the fruit set. For instance, inbred lines of cucumber resulted in a higher number of parthenogenetic embryos than hybrid lines (Niemirowicz-Szczytt et al., 1995). On the other hand, the growing environment is another key element to take into account when pollinating with irradiated pollen. During summer/spring the fruit set and the number of embryos is usually higher than in winter/autumn (Sauton, 1988; Hooghvorst et al., 2020).

Embryo Detection and Rescue

The use of irradiated pollen to pollinate allows the production of fruits potentially containing parthenogenetic embryos in some of their seeds. However, the vast majority of seeds are empty (Hooghvorst et al., 2020). Therefore, before embryo rescue, embryo have to be detected to be excised from the seed and cultured *in vitro*. Three different methods can be used to detect and rescue the parthenogenetic embryos: inspection of seeds one-by-one, X-ray photography and culture of seeds in liquid medium. Each one differs in the time invested, the efficiency and the required equipment (**TABLE 3.4.1**). The inspection of seeds one-by-one with the help of

a binocular microscope is the most widely-used method because it does not require specialized equipment and successfully detects parthenogenetic embryos. Moreover, a light box can be used to ease the inspection of seeds (Hooghvorst et al., 2020; Baktemur et al., 2013). Nevertheless, the inspection of seeds one-by-one is very laborious and time-consuming. On the other hand, the X-ray radiography is the most straightforward method due to is much faster than the inspection of seeds, but requires specialized equipment which is not always available in all laboratories (Claveria et al., 2005). Lastly, the culture of seeds in liquid medium has been frequently shown to fail, due to contamination with endophytic bacteria and fungi (Hooghvorst et al., 2020; Dong et al., 2016).

TABLE 3.4.1. Summary of the parthenogenetic methodology and efficiency in cucurbit species for doubled haploid (DH) line production.

Species	Embryo detection method	Embryos per fruit	Mortality rate *in vitro*	Ploidy-level	Chromosome doubling		Reference
					Method	Efficiency	
Cucumber	X-ray	0.23	68.23%	62% H 38% M	E20H8 medium suppl. 500 µM colchicine for 48 h	55% M 30% DH	Claveria et al. (2005)
Cucumber	One-by-one	5.79	79.73%	-	-	-	Smiech et al (2008)
Melon	One-by-one	6.27	30.85%	73% H 27% M	*In vitro* solution 500 $mg \cdot L^{-1}$ colchicine for 3 h	26% DH	Lim and Earle (2008)
Melon	X-ray	0.3	50.94%	73.08 H 23.08% DH 3.84% M	*In vivo* solution 5000 $mg \cdot L^{-1}$ colchicine for 2 h	9.38% M 20.31% DH	Hooghvorst et al (2020)
Melon	One-by-one	1.97	34.22%	90% H	E20H8 medium suppl. 500 µM colchicine for 48 h	-	Gonzalo et al. (2011)
Melon	-	-	-	-	*In vivo* solution 0.5% colchicine for 2 h	46.03% DH	Solmaz et al. (2011)

Pumpkin	One-by-one	16.38	84.04%	5.9% H	-	-	Kurtar et al. (2009)
Pumpkin	One-by-one	69.85	-	0.86% H			Košmrlj et al. (2013)
Squash	One-by-one	0.2-10.5	71.2 - 80.1%	43.7% H 56.3% D	-	-	Kurtar et al. (2002)
Squash	One-by-one	18.45	-	0.66% H			Baktemur et al. (2014)
Watermelon	One-by-one	1.4	-	-	-	-	
Winter squash	One-by-one	13.72	85%	10.96% H	-	-	Kurtar and Balkaya (2010)

In Vitro *Culture*

The detected parthenogenetic embryos are rescued and cultured *in vitro* in specific media. Several media can be used to culture embryos *in vitro* successfully such as E20A medium (Sauton and Dumas, 1987), MS (Murashige and Skoog, 1962), N6 (Chu, 1975) and CP (Chée et al., 1992). Nevertheless, E20A medium with or without modifications is the most commonly used medium for parthenogenetic embryo culture. The parthenogenetic haploid embryos have shorter and irregular cotyledons in comparison to diploid embryos. In addition, they can present a range of morphogenic shapes and stages (pointed, globular, arrow-tip, torpedo, heart, cotyledon, amorphous or necrotic). The survival, germination and development of parthenogenetic embryos is usually correlated with the shape and if its white or necrotic (Kurtar and Balkaya, 2010). In addition, during the *in vitro* process there is a high selection pressure because of deleterious gene combination in homozygosis that can be responsible of vegetative growth problems and can hamper the germination and plantlet development (Don't et al., 2016; Lim and Earle, 2008). Then, the germination of embryos and the growth and development of the parthenogenetic plantlets is not always guaranteed. The mortality rate of embryos and plantlets *in vitro* is dramatically high, 30–85% (**TABLE 3.4.1**).

Chromosome Doubling

Plantlets that survive parthenogenesis are usually haploid (~70%), but mixoploid or even spontaneous DHs can be obtained too (Hooghvorst et al., 2020; Dong et al., 2016). Spontaneous DH plantlets do not need to

undergo chromosome doubling and can be directly acclimatized for DH seed recovery. On the other hand, haploid and usually mixoploid plantlets have to be chromosome doubled with antimitotic compounds. In cucurbit species, chromosome doubling has been reported chiefly in melon and cucumber. The doubling treatment can be *in vitro* or *in vivo*. The antimitotic compound can be colchicine, oryzalin or trifluralin. However, it is mostly performed using colchicine. Dinitroanilines, oryzalin and trifluralin, have been reported as successful and very promising in cucumber (Ebrahimzadeh et al., 2018) and unsuccessful in melon 'Piel de Sapo' (Hooghvorst et al., 2020). A high mortality might be recorded after the treatment, due to the toxicity of colchicine or the hyperhidricity suffered by the explants. On the other hand, *in vivo* treatment applies higher concentration of colchicine for a shorter time (2–3 h) by immersing the apical meristems of plants growing in the greenhouse into the colchicine solution. Chromosome doubling is highly influenced by the genotype and the most suitable method must be determined empirically. In melon, the efficiency of chromosome doubling in "Piel de Sapo" type genotype was higher when applying colchicine at 5000 $mg \cdot L^{-1}$ for 2 h *in vivo* rather than at 500 $mg \cdot L^{-1}$ for 12 h *in vitro* (Hooghvorst et al., 2020), or in a BC4F1 population, the 26% of haploids chromosome doubled when exposing shoot tip explants to 500 $mg \cdot L^{-1}$ for 3 h *in vitro* (Lim and Earle, 2008); in cucumber, colchicine was applied *in vitro* in solid E20H8 medium for 48 h or by submerging plantlet nodes and tips into a colchicine solution for 3–12 h reaching a 55% of chromosome doubling (Claveria et al., 2005) (**TABLE 3.4.1**).

DH Seed Recovery

Pure and viable seed must be recovered from chromosome doubled and the spontaneous DHs plants. Those plants present a low fruit set (~3–10%) when are self-crossed due to a low germination ability of pollen and the presence of different ploidy-levels in the whole plant (Lim and Earle, 2009). Abnormal ploidy-level in the same plant can be observed in the female and male flowers during the process. The self-crossing must be by hand-pollination to avoid external pollinations. In addition, the germination of DH seed is difficult and a generation for seed multiplication is usually required prior to F1 hybrid seed production.

GENOME EDITING IN CUCURBIT SPECIES

Genome editing techniques have the ability to introduce mutations in the plant genome. There are three main site-specific genome-editing nucleases with the capacity to target precise regions of the genome: zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats associated to nuclease Cas9 (CRISPR/Cas9). The CRISPR/Cas9 system has risen as the preeminent genome editing technique, due to its versatility, efficiency and ease to engineering in comparison to ZFN and TALENs. In cucurbits, there are no reports describing the application of ZNF nor TALENs, as far as the authors know. Nevertheless, CRISPR/Cas9 has been applied successfully in cucumber (Hu et al., 2017; Chandrasekaran et al., 2016), watermelon (Tian et al., 2017, 2018; Zhang et al., 2020) and melon (Hooghvorst et al., 2019).

For the success of a CRISPR/Cas9 experiment, breeders need the sequenced genome of the target specie available, an adequate *Agrobacterium*-mediated transformation protocol and an efficient binary vector containing the sequence of the Cas9 protein capable to induce target mutations. In cucurbit species, the genomic sequences of cucumber, watermelon, melon, pumpkin, zucchini, squash, winter squash, bottle gourd and others are available (CuGenDB). Several CRISPR/Cas9 binary vectors have been reported successful in cucumber, watermelon and melon, and may be used for other cucurbits (**TABLE 3.4.2**). However, *Agrobacterium*-transformation protocol is still the main bottleneck to apply genome editing in cucurbits (Zhang et al., 2020; Hooghvorst et al., 2019).

The production of genome-edited plants in cucurbit species usually starts with the selection of the gRNAs targeting the gene of interest and the construction of the CRISPR/Cas9 binary vector. The CRISPR/Cas9 vector containing the gRNAs should be tested in protoplast prior transformation in order to corroborate the gene editing. The verification of the mutation induction before the *Agrobacterium*-mediated transformation process may ensure its success.

Agrobacterium-*Mediated Transformation*

The regeneration of transgenic plants in cucurbit species is considered a very recalcitrant process (Tian et al., 2017; Hooghvorst et al., 2019; Castelblanque et al., 2008). To successfully obtain of transgenic plants,

regeneration and transformation have to take place. In cucurbits, cotyledonary explants are the main source of plant material for direct organogenesis, which is the production of adventitious buds or shoots from explants without a callus phase. Organogenesis is usually high in cucurbit species when using a suitable regenerating medium. Nevertheless, transformation, the acquisition of the transgene by the germinative cells, is highly inefficient. Therefore, during the process of *Agrobacterium*-mediated transformation, many plants regenerate but the vast majority lacks the transgene. The non-transformed regenerants (so-called "escapes") frequently grow into selective medium, contrary to what occurs in other species. The percentage of "escapes" can be ~30% and are a hindrance in order to select positive transformants. Moreover, the transformation process is usually optimized for and restricted to a few genotypes. This impedes the application of transformation and genome editing to a wide range of genotypes of interest. In comparison to the transformation efficiency of other species such as rice, Arabidopsis or tomato, cucurbit species have a low transformation of 1.32–5.63% (**TABLE 3.4.2**).

TABLE 3.4.2. Summary of the methodology and efficiencies of CRISPR/Cas9 reported experiments in cucurbit species.

Species	Target Gene	Plant Material	Transformation		Genome Editing Efficiency	Reference
			Method	Efficiency		
Cucumber	*eIF4E*	Cotyledonary explants	*Agrobacterium*-mediated	-	20%	Chandrasekaran et al. (2016)
Cucumber	*WIP1*	Cotyledonary explants	*Agrobacterium*-mediated	1.32%	65.2	Hu et al. (2017)
Watermelon	*PDS*	Cotyledonary explants	*Agrobacterium*-mediated	1.67%	100	Tian et al. (2017)
Watermelon	*PDS*	Protoplasts	Protoplast transfection	-	42.1-51.6%	Tian et al. (2017)
Watermelon	*ACS*	Cotyledonary explants	*Agrobacterium*-mediated	-	23%	Tian et al. (2018)
Watermelon	*PSK1*	Cotyledonary explants	-	2.30%	-	Zhang et al., 2020
Melon	*PDS*	Cotyledonary explants	*Agrobacterium*-mediated	5.63%	42-45%	Hooghvorst et al. (2019a)
Melon	*PDS*	Protoplasts	Protoplast transfection	-	25%	Hooghvorst et al. (2019a)

In addition, the ploidy-level of T0 generation can be spontaneously duplicated during the direct organogenesis resulting in tetraploid regenerants. Those polyploid plants are sterile and selfing is impossible for segregation of the transgene or for transgenic seed multiplication.

Genome Editing Efficiency

The mutation efficiency depends on the GC content of the sgRNA, the number of transformed cells and the Cas9 protein expression level in transgenic cells (Ren et al., 2019). In species such as rice or Arabidopsis, the efficiency frequently ranges between 80–100% and in cucurbit species it ranges from 20–100% (**TABLE 3.4.2**). Taking into account the larger effort invested in rice or Arabidopsis to apply and optimize CRISPR/Cas9 in comparison to cucurbit species, CRISPR/Cas9 efficiency in cucurbits can be recognized as acceptable and suitable. Then, genome editing efficiency is not a bottleneck when applying CRISPR/Cas9 in cucurbits. Nevertheless, further attempts should be assayed, as done in other species, to achieve a higher genome editing efficiency such as using endogenous promoters of Cas9 and sgRNA expression, heat treatment during transformation, optimization of transformation efficiency (LeBlanc et al., 2018; Yan et al., 2015).

HAPLOID INDUCER-MEDIATED GENOME-EDITING IN CUCURBIT SPECIES

Haploid inducer approach is based on an intraspecific cross between a haploid inducer line and a receptor genotype of interest from where to obtain haploid lines. The haploid inducer line carries a specific mutation in an essential gene for the normal fertilization of female cells and therefore induces the parthenogenetic development of haploid embryo from the egg cell. The *MATRILINEAL* (*MATL*) gene, also known as *NOT LIKE DAD* (*NLD*) or *PHOSPHOLIPASE A1* (*PLA1*) encodes a pollen-specific phospholipase and is usually the mutated gene that causes the haploid inducer ability in cereals (Gilles et al., 2017; Liu et al., 2017; Prigge et al., 2012). For instance, in maize, natural haploid inducer lines were discovered to carry a 4-bp insertion in the carboxy terminus of the *MATL* gene. Natural haploid inducer lines have been used in wheat, maize, tobacco or barley for years to induce a parthenogenetic process for further haploid line obtention (Prigge et al., 2012; Laurie and Bennett, 1988; Coe, 1959; Burk et al., 1979; Kasha and Kao, 1970). In cucurbit species, no natural haploid inducer lines have been described, as far as the authors know. However, with the recent genome editing tools available, breeders have the possibility to generate haploid inducer lines through genome-editing techniques. Haploid inducer lines have been produced in cereal species mutating *MATL* gene via CRISPR/Cas9. Haploid inducer-mediated genome-editing approach yielded a 6.7% of parthenogenetic haploids in maize (Kelliher et al., 2017) and ~6% in rice (Yao et al., 2018). Those rates represent a great improvement in *in vivo* doubled haploid obtainment in those species to allow the acceleration of breeding.

The *MATL* gene is present and highly conserved in cereal species but not in dicots. Therefore, the haploid inducer-mediated genome-editing system in dicots usually targets centromeric histone 3 (*CENH3*) gene. The *CENH3* gene codes a histone present in all plants that determines the position of the centromere, and, thus, plays a major role in chromosome segregation during mitosis (Britt and Kuppu, 2016). The haploid inducer related potential of *CENH3* gene was first discovered in Arabidopsis. In this study, a haploid inducer line named "green fluorescent protein (GFP)-tailswap" carried GFP fused to the N-terminal tail domain of an H3 variant, replacing the N-terminal tail of *CENH3*. This *CENH3* mutant line produced a haploid induction rate of 25–45% when crossed with a *CENH3* wild type line by chromosomes elimination of the mutant line (**TABLE 3.4.3**) (Ravi and Chan 2010). Since the evidence of the potential of *CENH3* for haploid induction, some applications have raised

using ethyl methane sulfonate (EMS) mutagenesis targeting *CENH3* gene to obtain haploid inducing lines, such as in tomato with a haploid progeny of 0.2–2.3% (WO 2017 200386/KEYGENE); in cucumber, with an efficiency of 1%; and in melon, with an efficiency 1.5% (WO 2017 081,011 A1/RIJK ZWAAN) (**TABLE 3.4.3**). However, those procedures are patented, and EMS mutagenesis is quite inefficient in those species.

TABLE 3.4.3. Haploid induction reports mediated by mutation or disruption of centromeric histone 3 (*CENH3*), MATRILINEAL (*MATL*) or *DMP*.

Species	**Common name**	**Target gene**	**Haploid induction rate**	**Reference**
Monocotyledonous				
Triticum aestivum	Wheat	*MATL*	18.9%	Liu et al. (2019)
Oryza sativa	Rice	*MATL*	2-6%	Yao et al. (2018)
Zea mays	Maize	*DMP*	0.1-0.3%	Zhong et al. (2019)
Dicotyledonous				
Arabidopsis thaliana	Arabidopsis	*DMP*	2.1%	Zhong et al. (2020)
Arabidopsis thaliana	Arabidopsis	*CENH3*	1.1-44%	Kuppu et al. (2020)

Therefore, the generation of haploid inducer lines in cucurbit species may be democratized targeting *CENH3* using CRISPR/Cas9, due to the specificity of CRISPR/Cas9 in multiple sequences of the *CENH3* gene which can be targeted efficiently. However, for the generation of haploid inducer lines mutated via CRISPR/Cas9 a successful transformation protocol would be imperative at least for one genotype. Generally, transformation protocol is adjusted for a specific genotype in cucurbit species. Fortunately, the genotype of the haploid inducer line is not an issue for haploid induction as long as it is the same species as the donor genotype from where to recover haploid embryos and bears male flowers. From the T0 CRISPR/Cas9 generation, heterozygous *CENH3*-mutated should be self-crossed in order to select: (i) T1 transgene-free and homozygous *CENH3*-mutated

individuals, and (ii) transgene-free and heterozygous mutated-*CENH3* T1 individuals. The homozygous *CENH3*-mutated lines will be used for haploid induction process and heterozygous lines for haploid inducer line maintenance for future applications. Through this method, haploid inducer lines may be successfully maintained and used over the time (**FIGURE 3.4.1**).

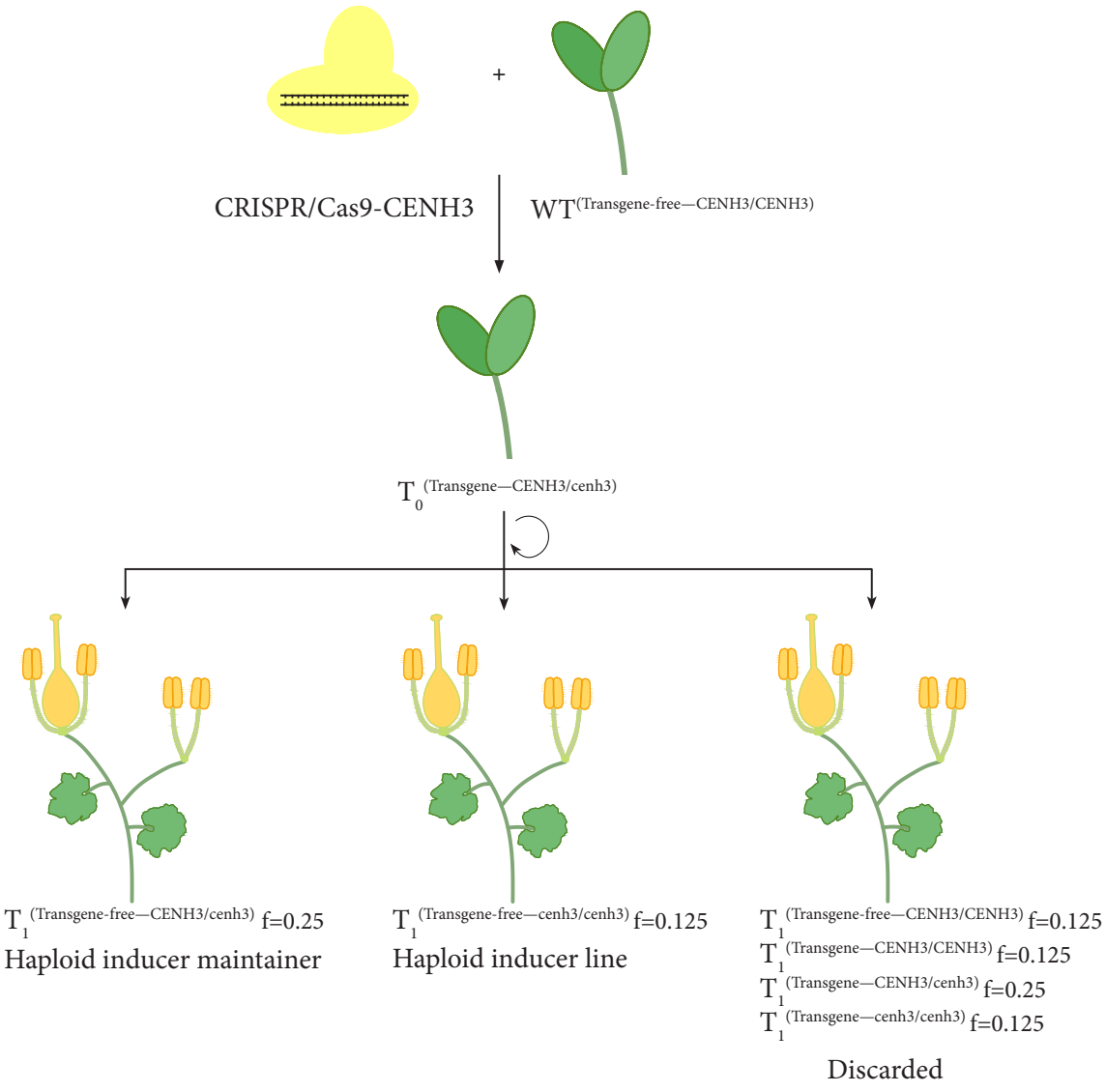

FIGURE 3.4.1. Schematic representation of the obtention of a haploid inducer line and its maintainer. WT: wild type.

The application of the haploid inducer-mediated genome-editing system in cucurbit species could reduce the actual time-consuming, labor-intensive and limited parthenogenic process. Although the process would remain very similar to parthenogenetic process with irradiated pollen due to the initial *in vivo* pollination with pollen of the haploid inducer line, line, the rate of haploid embryo induction could be significantly improved since no

aggressive treatment, such as ionization, would be applied in haploid inducer line pollen. Furthermore, no irradiation source requiring equipment not always available in all laboratories will be needed. On the other hand, parthenogenesis via irradiated pollen is highly genotype-dependent and accomplishment of irradiation depends upon the radiation resistance of the pollen. In addition, some genotypes are reported to be very recalcitrant to induction of haploid generations when pollinated with irradiated pollen. Pollination with pollen of the haploid inducer lines could broaden the range of genotypes that can produce haploids and increase the number of parthenogenetic embryos. This must be assayed to see whether haploid inducer lines can increase the number of haploid embryos produced in cucurbits.

Moreover, parthenogenesis with irradiated pollen is routinely applied in cucumber and melon and substantial progress has been made. In contrast, less effort has been applied in species such as watermelon, winter squash, pumpkin or bottle gourd and, therefore, less progress has been made in optimizing their parthenogenetic protocols. In cucurbit species, the fruits set once pollinated with irradiated pollen or pollen from haploid inducer lines will follow the same steps as in *in situ* parthenogenesis via irradiated pollen. Consequently, limiting factors described for parthenogenesis with irradiated pollen would likewise be present using the haploid inducer approach. Therefore, the haploid inducer-mediated genome-editing approach can take advantage of the progress made to successfully obtain doubled haploid lines.

The haploid inducer-mediated genome-editing approach is an opportunity to improve the efficiency of doubled haploid production in recalcitrant species. The use of *matl* or *cenh3* mutant lines in cereals or dicots for haploid induction and production might avoid androgenesis, gynogenesis or parthenogenesis via pollen irradiated and boost their obtention in several species.

REGULATORY LANDSCAPE FPR THE NEW GENERATION OF DOUBLED HAPLOIDS

The development of DH lines has never been restricted by regulatory limitation, because they are obtained using *in vitro* protocols for haploid generation or *in vivo* pollination with pollen mutagenized with gamma rays. On the other hand, since the Directive 2001/18/EC put in place in EU, GMOs have been strongly limited because of uncertainty and safety. The GMO definition is "as an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination". The European highest court handed a setback regulation on 25 July of 2018, subjecting organisms obtained using CRISPR/Cas9 and other genome editing techniques under the same regulation as GMOs, defining them as "recombinant nucleic acid techniques involving the formation of new combinations of genetic material". Therefore, GMOs and CRISPR/Cas9-derived plants have the same regulation landscape despite the fact that the first added a transgene essential for the trait improvement whereas the second used recombinant DNA for trait improvement and subsequently lost it through segregation. Then, as pointed out by Abbot, the EU criterion is based on the process and the product when using recombinant DNA (Abbot, 2015).

Doubled haploids derived from haploid inducer-mediated genome-editing approach presents a challenge in terms of regulation. When haploid lines are produced by crossing the haploid inducer line with a receptor genotype, the chromosomes of the haploid inducer line are eliminated, and the haploid-derived progeny carries the maternal set of chromosomes. The only progenitor of the haploid generation has never carried recombinant DNA and has no improved traits derived from mutations induced by the genome editing technique. The origins of haploid lines derived from haploid inducer or irradiated pollen are impossible to trace. Therefore, haploid-mediated genome-editing system should not be restricted by any limitation or regulation.

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4. GENERAL DISCUSSION

4.1. DOUBLED HAPLOIDS

4.1.1. Doubled haploid uses

DH lines are highly important for plant breeding due to their complete homozygosis, making qualitative and quantitative phenotypic selection more efficient. Since the very first attainment of DHs in *Brassica napus* (Thompson, 1972), many publications have reported the development of DH lines in more than 250 species (Maluszynski, 2003). Following the research conducted in the 70s and 80s that demonstrated the ability to generate DHs in many cereal, vegetable and horticultural crops, the focus in recent decades has been optimizing and assaying different ways to enhance DH production in each species and genotype by introducing changes in every step of the DH programs.

DHs have been of great importance for: establishing chromosome maps and whole genome sequencing in the vast majority of genetically mapped and sequenced species; bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations; and, for mapping quantitative trait loci (QTLs) (Forster et al. 2007). This usage of DHs in basic research has been extended into direct application in variety improvements. Furthermore, DHs are used as commercial cultivars such as stabilized homozygous lines or as parental lines to produce F1 hybrid lines, avoiding classical breeding methods to obtain stabilized and non-segregant lines.

4.1.2. Strategies for the production of doubled haploid material

Gametic haploid cells are the initial material used to obtain DH lines. Gametes from meiotic cells allow the generation of plantlets when cultured *in vitro* or when pollinated with irradiated pollen. The haploid step can be either a microspore from an anther or an ovule from an ovary depending on the species. The usual methods to induce haploids are as follows: androgenesis, gynogenesis and parthenogenesis (**FIGURE 4.1**). Plantlet regeneration from microspores or ovaries is a two-step protocol if a callus step is required prior to plantlet

regeneration, or a one-step protocol if it directly induces an embryo or regenerates a plantlet. Besides, gametic cells from meiosis can be developed into haploid embryos, via parthenogenesis. Thus, a DH process always requires a gametic haploid step from which haploid or DH plantlet will be regenerated.

In vitro culture techniques for gametic cells in androgenesis and gynogenesis techniques allow the original gametophytic pathway of the gamete be redirected towards a sporophytic pathway where plantlets can be regenerated. Androgenesis is the most common method to produce DHs (**TABLE 4.1**). Isolated microspores or microspores contained in anthers are cultured in specific induction media to induce the formation of callus. Subsequently, these calli are cultured in regeneration media to regenerate fully formed plantlets. In most cereal species, androgenesis is the only or the best method for DH generation with a high rate of spontaneous doubling, in species such as: rice (Hooghvorst et al., 2018), oat (Kiviharju et al., 2017) and bell pepper (Keleş et al., 2015). Gynogenesis stimulates *in vitro* embryogenesis development of the unfertilized haploid egg cells. In this process, a two-step protocol is usually carried out to induce callus formation from the female ovules in induction medium and to regenerate plants from callus in regeneration medium. For onion (Fayos et al., 2015) or beet (Hansen et al., 1995), gynogenesis is the best method for DH production. The ploidy level of the androgenetic and gynogenetic regenerated plants can differ depending on the cell events related to spontaneous or induced chromosome doubling (see below). Haploids, doubled haploids, mixoploids and tetraploids can be produced during the *in vitro* DH process (**FIGURE 4.1**). In androgenesis and gynogenesis it is desirable that the regenerated plantlets originate from microspore or ovule cells; nonetheless, somatic embryogenesis from anther or ovary tissues can take place. This process is defined as the regeneration of a whole plant from undifferentiated somatic cells in *in vitro* culture. The ploidy of these plantlets is diploid, and the genomic background is identical from which DHs are expected to be generated (**FIGURE 4.1**). The parthenogenesis methodology allows the formation of an embryo from an egg cell without fertilization. Egg cells can be induced to develop into haploid embryos following *in situ* pollination with irradiated pollen, and these embryos only inherit the maternal set of chromosomes due to false fertilization. Such embryos germinate *in vitro* and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants. In the Cucurbitaceae, parthenogenesis is the only successful approach to obtain DH plant material (Dong et al., 2016).

Conventional DH technology has had great importance in some species to produce pure homozygous lines. Nonetheless, in all reported species there is a high genotypic dependency on the efficiency of the method, with some cultivars adapted to the existing protocols and some others very recalcitrant to the process. Publications on rice, wheat and maize show correlation with the substantial progress in haploid technology, attainable given the intensive research efforts (Croser et al., 2007). The majority of crops have acceptable DH protocols from which DH lines are produced successfully, but they require a significant time investment that ranges from five months to two years, substantial personnel and equipment needs, and always have inevitable variability in efficiency, depending on the genotype used. There are even some crops of great economic importance, including tomato species (Seguí-Simarro et al., 2011) and members of the Cucurbitaceae and Fabaceae family (Croser et al., 2007; Dong et al., 2016), that lack a successful beginning to end protocol for DH production.

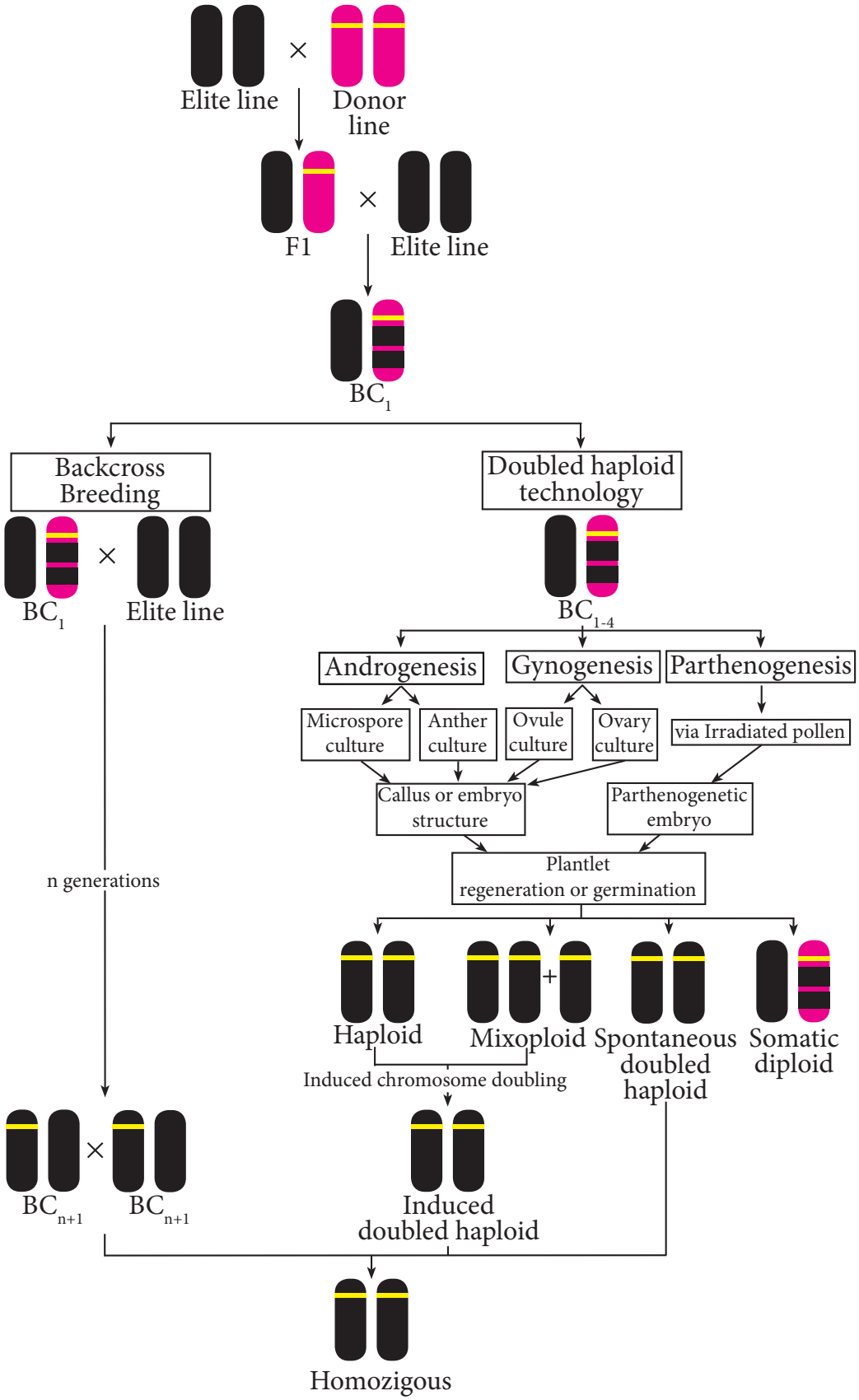
Elite line
Donor line
F1
Elite line
BC1
Backcross Breeding
Doubled haploid technology
BC1
Elite line
BC1-4
Androgenesis
Gynogenesis
Parthenogenesis
Microspore culture
Anther culture
Ovule culture
Ovary culture
via Irradiated pollen
Callus or embryo structure
Parthenogenetic embryo
n generations
Plantlet regeneration or germination
Haploid
Mixoploid
Spontaneous doubled haploid
Somatic diploid
Induced chromosome doubling
BCn+1
BCn+1
Induced doubled haploid
Homozigous

FIGURE 4.1. Schematic representation of backcross breeding and doubled haploid technology. Retrieved and modified from (Hooghvorst and Nogués, 2020).

4.1.3. Approaches and process of the chromosome-doubling step

Every DH program starts with the haploid gametophytic phase to efficiently obtain DH plants. During the latter part of the *in vitro* process, haploid plant material needs to undergo chromosome duplication to finally obtain a fertile plant, from which DH seeds are recovered. The original chromosome set, whether maternal (gynogenesis and parthenogenesis) or paternal (androgenesis), must go through a spontaneous or induced duplication. The effective duplication of the haploid material is essential for the success of the DH process, because haploid plants are infertile. The chromosome doubling can be spontaneous or induced. Earlier duplication is the ideal for avoiding mixoploid plants, or fully haploid plants, and to ensure a battery of DH plants.

Endomitosis and nuclear fusion are the main causes of spontaneous duplication. These processes have been extensively studied in barley and inferred in other species (Kasha, 2005). During mitosis, chromosome multiplication and separation of cells usually occurs. Instead, in endomitosis, multiplication occurs but the cell fails to divide and one nucleus with two sets of chromosomes is restored. During nuclear fusion, two or more synchronized nuclei divide and develop a common spindle. Spontaneous chromosome doubling capacity during the process depends on the species and genotype. For example, the frequency of spontaneous DH androgenic bell pepper plants is 30-55% (Irikova et al., 2011; Keleş et al., 2015); in rice, it ranges between 8 to 30% of the regenerated plants (Alemanno and Guiderdoni, 1994; Hooghvorst et al., 2018; López-Cristoffanini et al., 2018). In species whose spontaneous doubling rate is high, the process of induced chromosome doubling has not been explored largely for obvious reasons. Nevertheless, despite this, some genotypes are more likely to regenerate spontaneous DHs than others because all *in vitro* processes are genotypically dependent, including duplication. Indeed, species with generally high rates of spontaneous DH still need efficient protocols to induce chromosome doubling with antimitotic compounds because some genotypes within such species have low rates of spontaneous DH generation or no spontaneous production at all, meaning that antimitotic application is still essential.

A proportion of the so-called 'spontaneous' duplication reported in the literature is actually induced via chromosome doubling by means of pre-treatments like heat or cold pre-treatments that do not involve antimitotic. Temperature stress, like heat or cold pre-treatments, is usually applied during androgenesis and gynogenesis prior to *in vitro* culture. Many of these pre-treatments were originally intended to increase microspore induction, but

they usually increase the frequency of chromosome doubling due to the destabilization of microtubules and microfilament elements that form the cytoskeleton (Kasha, 2005). In microspores, cold pre-treatment is related to failure of cell wall formation leading to multinucleate structures (known as coenocytic structures, see Testillano et al. (2002)) which result from nuclear division without cytokinesis. However, in spite of the basic research that has related pre-treatment protocols to increased number of microspores at the optimal stage, once the microspore culture is started and plants regenerate, it is difficult to demonstrate that the pre-treatment is the causal agent of the increase in frequency of DHs beyond the determination of the best microspore stage for embryogenesis.

Induced chromosome duplication may be feasible at different stages of the process: at the first pollen mitotic division of microspore cells, at the callus stage when growing *in vitro*, or at the plant stage when regenerated (**TABLE 4.1**). The use of antimitotic compounds is mandatory when spontaneous chromosome doubling is absent or very low. A specific type of endomitosis, known as C-mitosis, takes place when antimitotic compounds are used to destabilize the cell cycle, perturbing not only mitosis but also arresting cells during interphase (Lu et al., 2012). In interphase, DNA is replicated and each replicated chromosome forms sister chromatids that are bound by the centromere's spindle tubules. When C-mitosis occurs, the antimitotic compound interacts with tubulin subunits destabilizing and inhibiting their assembly. Antimitotic treatment depends on the species and the protocol used for obtaining DH plants. Some key features considered are the antimitotic agent, its concentration, the exposure time and the treatment stage, which were thoroughly reviewed by Dhooghe et al. (2009).

In androgenetic protocols for DH production, free-microspores or microspores contained in anthers are usually cultured in colchicine-supplemented medium. By this means, chromosome doubling is achieved at earlier stage ensuring the success of the process. In addition, an increased level of microspore induction has been described attributed to the presence of colchicine (Alemanno and Guiderdoni, 1994; Iqbal et al., 1994; Barnabás et al., 1999; Hooghvorst et al., 2018).

Haploid plants as explant material are another important source for recovering DHs. Most of the DH protocols apply antimitotic compounds to plants grown either *in vitro* or *in vivo* to achieve chromosome doubling (**TABLE 4.1**). Immersion of the whole *in vitro* plantlet or the apical meristem *in vivo* are two approaches that usually yield good chromosome doubling results in many plant species (**TABLE 4.1**). *In vitro* treatments usually

take longer and the antimitotic concentration is lower, whereas *in vivo* treatments have shorter exposure times with higher concentrations of compound. Nevertheless, in some species like onion, the apical meristem of adult plants is inaccessible, impeding chromosome doubling *in vivo* (Bohanec, 2002). Meanwhile, during propagation of regenerated haploid plants chromosome doubling has also been achieved by adding antimitotic compounds to the culture medium. Breeders sometimes discard androgenetic and gynogenetic haploid plants when spontaneous doubled regenerants are considered acceptable. Despite their great potential, the lack of a successful protocol for chromosome doubling in grown plants means that they frequently go to waste. In contrast, the parthenogenetic process usually depends on whole plants or embryos as the source of material for DH (**TABLE 4.1**).

Several antimitotic agents have been used for chromosome doubling of haploid plants. Colchicine is the most popular antimitotic agent used for DH studies in most species because it has a high chromosome doubling ability. Yet there are many different chemicals with antimitotic effects such as amiprophosmethyl (AMP), pronamide, propham, oryzalin and trifluralin, which have similar effect and mechanisms of action to colchicine (Bartels and Hilton, 1973; Lu et al., 2012). Colchicine is a toxic natural product extracted from plants of the Colchicum genus and used as an antimitotic agent. It is known to inhibit mitosis in a wide variety of plant and animal cells by interfering with the structure of the mitotic spindle (Eigsti and Dustin, 1955). Furthermore, research shows that the colchicine-binding protein is a subunit of microtubules. It has been reported that low dosages of the compound can effectively halt cell division for a small period of time thus producing a doubling of the genetic load in some cells (Borisy and Taylor, 1967). Nevertheless, colchicine has a highly toxic effect on plant and animal cells, being a hazardous compound for researchers to use in the laboratory. Moreover, when plants are treated with colchicine to induce doubling, the mortality rate is usually high, and is dependent on the concentration, time of exposure and species. Consequently, to optimize protocols, breeders need to balance dose and exposure time to ensure successful chromosome duplication while limiting the mortality rate.

Nowadays, oryzalin and trifluralin are being widely employed as substitute for colchicine. These compounds are dinitroaniline herbicides reported to bind to plant tubulin, which in turn confers an antimitotic effect similar to colchicine. Unlike colchicine, dinitroanilines have no effect on microtubules in vertebrate cells, which are resistant to its depolymerizing effects. It has been demonstrated that oryzalin has a high-affinity interaction with plant tubulin, binding rapidly and reversibly while forming a tubulin-oryzalin complex (Hugdahl

and Morejohn, 1993). The properties of dinitroaniline binding to tubulin are different from colchicine. In fact, dinitroaniline binding is time independent and the tubulin-dinitroaniline complex dissociates completely, unlike colchicine, which has been reported to bind slowly to tubulin and the tubulin-colchicine complex does not easily dissociate. Furthermore, oryzalin has been reported to have a much higher affinity for unpolymerized tubulin than the polymerized form (Borisy and Taylor, 1967; Hugdahl and Morejohn, 1993). Scientific interest lies in the fact that dinitroanilines can be much less hazardous for humans than colchicine and equally effective at lower doses.

Key features commented above for a successful chromosome doubling need to be determined empirically for each species. More work is required concerning the chromosome-doubling step in many species and genotypes, describing the best results of different treatments (**TABLE 4.1**).

The antimitotic compound is usually dissolved in dimethyl sulfoxide (DMSO). DMSO has a double utility, as a solvent to dissolve the antimitotic and to increase cell permeability by allowing an increase in absorption of the agent into the plant (Hamill et al., 1992). However, DMSO may increase plant mortality due to its relative toxicity (Dhooghe et al., 2011). Other solvents can be used instead, such as NaOH or 70% ethanol for oryzalin, acetone for trifluralin, or even water (Dhooghe et al., 2011). If chromosome doubling is performed by immersion of some part of the plant in an antimitotic solution, detergent or surfactants can be added too to enhance the surface contact. In contrast, if the treatment is applied to a specific area on the plant, such as the lateral or apical meristems, lanolin paste can be used to localize a dose of solution.

TABLE 4.1. Overview of the most commonly used methods for doubled haploid production and chromosome doubling and their efficiency in major crops.

Species	Common name	Doubled haploid method	Induced chromosome doubling method					Chromosome doubling efficiency[b]		Reference
			Stage	Application	Antimitotic compound	Concentration	Exposure time[a]	Spontaneous	Induced	
Cereal crops										
Avena sativa	Oat	Anther culture	*In vitro* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	37.42%	88.17%	Kiviharju et al. 2017
Hordeum vulgare	Barley	Microspore culture	-	-	-	-	-	>90%	88.3-93.5%	Li and Devaux, 2003
Hordeum vulgare	Barley	Anther culture	*In vivo* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	-	-	Jacquard et al. 2003
Sorghum bicolor	Sorghum	Anther culture	-	-	-	-	-	95.3%	-	Kumaravadivel and Sree, 1994
Secale cereale	Rye	Anther culture	-	-	-	-	-	13-67%	-	Tenhola-Roininen et al. 2005
Triticum aestivum	Wheat	Microspore culture	Tillers of *in vivo* plants	Immersion	Colchicine	0.10%	5-8 h	-	95.60%	Niu et al. 2014
Triticum aestivum	Wheat	Microspore culture	Internode of pollinated spikes	Injection	Colchicine	1% suppl. 100 ppm 2,4-D	48 and 72 h	0	33-100%	Sood et al. 2003
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	250 mg·L^{-1}	48 h	31%	65.50%	Alemanno and Guiderdoni, 1994
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	300 mg·L^{-1}	48 h	0-0.18 DH green plantlets per 100 anthers	0.75 DH green plantlet per 100 anthers	Hooghvorst et al. 2018
Oryza sativa	Rice	Anther culture	*In vitro* plantlets	Immersion	Colchicine	500 mg·L^{-1}	5 h	-	35%	Hooghvorst et al. 2020a
Triticum x *Rye*	Triticale	Microspore culture	Embryos	Microspore culture	Colchicine	0.3 mM	24 h	30%	50-55%	Würschum et al. 2012

Zea mays	Maize	Anther culture	Anthers	Induction medium	Colchicine	0.2 g·L^{-1}	3 d	19%	20%	Obert and Barnabás, 2004
Zea mays	Maize	Anther culture	Microspores	Induction medium	Colchicine	1,250 µM	1 w	40%	93.75% DH plantlets for 100 anthers	Antoine-Michard and Beckert, 1997
Vegetable and horticultural crops										
Apiaceae or Umbelliferae										
Daucus carot	Carrot	Microspore culture	*In vivo* plants	Immersion	Colchicine	0.34%	1.5 h	50%	-	Ferrie et al. 2011
Pastinaca sativa	Parsnip	Microspore culture	*In vivo* plants	Immersion	Colchicine	0.34%	1.5 h	50%	-	Ferrie et al. 2011
Brassicaceae										
Brassica napus	Rapeseed	Microspore culture	Microspores	Induction medium	Colchicine	500 mg·L^{-1}	15 h	45-64.3%	83-91%	Zhou et al. 2002
Brassica oleracea var. *capitata*	Cabbage	Microspore culture	Rooted *in vitro* plantlets	Immersion	Colchicine	0.2% suppl. 2% DMSO	9-12 h	0-76.9%	58.3-75%	Yuan et al. 2015
Brassica oleracea var. *italica*	Broccoli	Microspore culture	Rooted *in vitro* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	6-12 h	50.6-100%	54.5-58.3%	Yuan et al. 2015
Cucurbitaceae										
Cucumis melo	Melon	Parthenogenesis	*In vivo* plants	Immersion	Colchicine	0.50%	2 h	-	46.03%	Solmaz et al. 2011
Cucumis melo	Melon	Parthenogenesis	*In vivo* plants	Immersion	Colchicine	0.50%	2 h	23%	20.93% DH and 9.30% mixoploids	Hooghvorst et al. 2020b
Cucumis sativa	Cucumber	Parthenogenesis	*In vitro* microcuttings	Culture medium	Colchicine	500 mg·L^{-1}	48 h	0%	30% DH and 55% mixoploid	Claveria et al. 2005
Cucumis sativa	Cucumber	Parthenogenesis	*In vitro* nodal explants	Immersion	Oryzalin	50 mg·L^{-1}	18 h	-	86.21%	Ebrahimzadeh et al. 2018
Solanaceae										
Capsicum annuum	Bell pepper	Anther culture	*In vitro* axillary buds	Lanolin paste in axillary buds	Colchicine	1%	-		n/a	Gyulai et al. 2000
Capsicum annuum	Bell pepper	Anther culture	-	-	-	-	-	22.2-53.4%	-	Keleş et al. 2015

Solanum melongena	Eggplant	Anther culture	*In vitro* axillary buds	Lanolin paste in axillary buds	Colchicine	0.50%	-	60%	25%	Corral-Martínez and Seguí-Simarro, 2012
Solanum tuberosum	Potato	-	Nodal segment	Immersion	Oryzalin	25 µM	8 h	20-78%	10.10%	Greplová et al. 2009
Other important plant crops										
Allium cepa	Onion	Gynogenesis	Embryos	Solid elongation medium	APM	25 µM	24 h	-	35%	Fayos et al. 2015
Allium cepa	Onion	Gynogenesis	*In vitro* plantlets	Culture medium	Colchicine	10 mg·L^{-1}	3 d	1%	46%	Campion et al. 1995
Allium cepa	Onion	Gynogenesis	*in vitro plantlets*	Media culture	Oryzalin	10 µM	3 d	-	67%	Jakše and Bohanec, 2003
Asparagus officinalis	Asparagus	Anther culture	*In vitro* shoot tips	Apical lanolin application	Colchicine	1.20%	-		21-97%	Tsay, 1997
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	Colchicine	0.40%	2.5 h	7.10%	4 DH per 100 ovules	Hansen et al. 1995
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	AMP	100 µM suppl. 1.5% DMSO	5 h	6.60%	4.7 DH per 100 ovules	Hansen and Andersen, 1998
Beta vulgaris	Beet	Ovule culture	*In vitro* plantlets	Agarose culture medium	Trifluralin	3.4 mg·L^{-1}	36 h	0-10%	62.50%	Gürel et al. 2000
Nicotiana tabacum	Tobacco	Anther culture	*In vivo* plant	Root dipping	Colchicine	0.50%	24 h		21-32%	Sood et al. 2013

[a] *h* hours, *d* days and *w* weeks

[b] If not specified, percentages refer to chromosome doubled material relative to the initially treated material

4.1.4. Ploidy-level identification of plant material

Ploidy identification of the plants produced during the DH process is essential before the chromosome-doubling treatment to determine whether the plant material has undergone spontaneous duplication, and afterwards to check whether or not the antimitotic treatment has successfully doubled the plants' chromosomes. There are multiple methods to check ploidy level: cytologically, morphologically, via marker-assisted selection or via flow cytometry method.

Cytological procedures for ploidy level determination can be carried out by counting chromosomes or examining the epidermal tissue of the leaves. Chromosome counting usually uses root tip cells, which are fixed, and the chromosomes are then stained and observed for counting (Maluszynska, 2003). Cytological analysis of leaves correlates chloroplast number, stomata dimensions and size with ploidy level. However, applying chromosome-counting methods to species with small chromosome size is very time-consuming and difficult, and chloroplast and stomata analysis is species- and genotype-dependent. Despite this, results from both cytological procedures are extremely accurate and sample preparation and staining is easy and fast.

Haploid and diploid plants can also be distinguished according to morphological observations of the plant material. Morphological observation is based on comparing plant traits of the donor plants and regenerants, such as: height, vigour, leaf shape, flower development, fertility and presence of pollen. This methodology does not require special equipment, but it is sometimes unreliable and subject to environmental effects. In the *Cucurbitaceae* family, leaf morphology, flower shape and size, pollen production, stem length or node number, are phenotypic variations that can be analysed for ploidy determination (Dong et al., 2016). Couto et al. (2013) correlated haploid levels with small plant size and brittle leaves, but excluded ploidy determination of haploid seeds via morphometric parameters due to the great variability in the seeds. Yuan et al. (2015) detected haploid individuals in two *Brassica* species due to the weak growth of the haploids and the small size of the plants as wells as the presence of smaller flower buds, the absence of pollen, and a lack of stamens in the flowers.

The use of segregating alleles in the donor parent is another methodology for ploidy level determination via marker-assisted selection. Simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) marker analysis techniques are ideal to identify ploidy-level as well as homozygosity in spontaneous DH regenerants or heterozygosity in diploid somatic regenerants. For instance, in soybean, 114 androgenic embryo-like structures were analysed for the Satt418 SSR marker, and 74% were found to be heterozygous originating from somatic anther tissue (Rodrigues et al., 2004). In pumpkin, 23 SSR markers were screened in 253 parthenogenetic diploid plants and the results showed no spontaneous chromosome doubling (Košmrlj et al., 2013). In oat, (Kiviharju et al., 2017) DNA markers were used for selection and this indicated that 3.4 % of regenerated androgenic plants were heterozygous.

Despite all the methods described above for ploidy-level determination, flow cytometry is the most used method for a wide range of species because of its convenience and rapidity estimating the nuclear genome size in plants (Doležel et al., 1998) . Estimation of ploidy-level via nuclear suspensions of plant cells and chromosome staining has been used in DH programs for routine laboratory analysis. Many optimizations have been done in order to increase the number of nuclei in suspension using extraction methods such as chopping and bead beating, alongside with numerous modifications of isolation buffers and staining procedures (Doležel and Barto, 2005; Hooghvorst et al., 2019). Moreover, in addition to haploid and diploid cells, flow cytometry is especially useful for detecting triploid, tetraploid and mixoploid plants. On the other hand, flow cytometry requires highly specialized equipment that is not required with earlier methods of ploidy determination.

4.1.5. Doubled Haploid Procedure in Cucurbits

Doubled haploid lines in cucurbit species can be produced by parthenogenesis, androgenesis or gynogenesis. However, parthenogenesis is the currently-predominant technique to produce doubled haploids in cucumber, melon, watermelon and pumpkins (Dong et al., 2016). The parthenogenesis process starts with *in vivo* pollination using irradiated pollen. The resulting parthenogenetic embryos are then

detected, rescued and cultured *in vitro*, in order to germinate and develop into plantlets. The ploidy-level of parthenogenetic plantlets is estimated by flow cytometry and can result in haploid, spontaneous doubled haploid, mixoploid or tetraploid levels. The haploid and mixoploid plantlets need to undergo *in vitro* or *in vivo* chromosome doubling, usually using colchicine as antimitotic compound. Once the plantlets have doubled their chromosomes, they are cultured in the greenhouse together with the spontaneously-doubled haploid lines to recover DH seed (Dong et al., 2016; Hooghvorst et al., 2020b; Lim and Earle, 2008). During the DH process, a high genotypic dependency and other factors continuously hamper each step causing a loss of efficiency that might be critical.

4.1.5.1. Pollination with Irradiated Pollen and Fruit Set

The first step of the *in situ* parthenogenetic process is the irradiation of mature pollen and the pollination of the receptor female flowers. γ-ray (^{60}Co or ^{137}Cs) and soft X-ray are the usual irradiation sources applied to male flowers. The ionized pollen can germinate on the female stigma and grow pollen tubes to reach the embryo sac. However, this pollen is genetically inactive and unable to fertilize the egg-cell and the polar nuclei. Therefore, irradiated pollen stimulates egg-cell division and parthenogenetic embryo induction (Hooghvorst et al., 2020b). Overall, the dose of ionizing radiation can range from 25 to 500 Gy, depending on the species and can yield less parthenogenetic haploid embryos at higher or more diploid embryos at lower dosages. Therefore, irradiation should be optimized for each species because pollen sensitivity is attributed to radiation-resistance.

The parameters that define the success or failure of pollination with irradiated pollen are the number of fruits set and the ratio of parthenogenetic embryos per fruit. Fruit set is lower when pollination is performed using irradiated pollen (Hooghvorst et al., 2020b). The number of female flowers that develop into a fruit can range between 10–25% in melon (Hooghvorst et al., 2020b), 20–25% in pumpkin (Košmrlj et al., 2013) or 50% in cucumber (Smiech et al., 2008). The initial number of parthenogenetic embryos is crucial to have enough plant material during the whole process. The plant material usually decreases progressively during each step of the DH process, due to mortality, inefficiency of the method

and recalcitrant performance. Frequently, the ratio of parthenogenetic embryos per fruit is low, 0.23–5.79 in cucumber (Claveria et al., 2005; Smiech et al., 2008); 0.2–16 in pumpkin, squash and winter squash (Košmrlj et al., 2013; Kurtar et al., 2002, 2009; Kurtar and Balkaya, 2010); 0.3–6 in melon (Dong et al., 2016; Hooghvorst et al., 2020b); or 1.4 in watermelon (Baktemur et al., 2014).

In addition, the genotype of the donor and the receptor plants have an influence in the fruit set. For instance, inbred lines of cucumber resulted in a higher number of parthenogenetic embryos than hybrid lines (Niemirowicz-Szczytt et al., 1995). On the other hand, the growing environment is another key element to take into account when pollinating with irradiated pollen. During summer/spring the fruit set and the number of embryos is usually higher than in winter/autumn (Hooghvorst et al., 2020b; Sauton, 1988).

4.1.5.2. Embryo Detection and Rescue

The use of irradiated pollen to pollinate allows the production of fruits potentially containing parthenogenetic embryos in some of their seeds. However, the vast majority of seeds are empty (Hooghvorst et al., 2020b). Therefore, before embryo rescue, embryo have to be detected to be excised from the seed and cultured *in vitro*. Three different methods can be used to detect and rescue the parthenogenetic embryos: inspection of seeds one-by-one, X-ray photography and culture of seeds in liquid medium. Each one differs in the time invested, the efficiency and the required equipment (**TABLE 4.2**). The inspection of seeds one-by-one with the help of a binocular microscope is the most widely-used method because it does not require specialized equipment and successfully detects parthenogenetic embryos. Moreover, a light box can be used to ease the inspection of seeds (Baktemur et al., 2013; Hooghvorst et al., 2020b). Nevertheless, the inspection of seeds one-by-one is very laborious and time-consuming. On the other hand, the X-ray radiography is the most straightforward method due to is much faster than the inspection of seeds, but requires specialized equipment which is not always available in all laboratories (Claveria et al., 2005). Lastly, the culture of seeds in liquid medium has been frequently shown

to fail, due to contamination with endophytic bacteria and fungi (Dong et al., 2016; Hooghvorst et al., 2020b).

4.1.5.3. In Vitro *Culture*

The detected parthenogenetic embryos are rescued and cultured *in vitro* in specific media. Several media can be used to culture embryos *in vitro* successfully such as E20A medium (Sauton and Vaulx, 1987), MS (Murashige and Skoog, 1962), N6 (Chu, 1978) and CP (Chée et al., 1992). Nevertheless, E20A medium with or without modifications is the most commonly used medium for parthenogenetic embryo culture. The parthenogenetic haploid embryos have shorter and irregular cotyledons in comparison to diploid embryos. In addition, they can present a range of morphogenic shapes and stages (pointed, globular, arrow-tip, torpedo, heart, cotyledon, amorphous or necrotic). The survival, germination and development of parthenogenetic embryos is usually correlated with the shape and if its white or necrotic (Kurtar and Balkaya, 2010). In addition, during the *in vitro* process there is a high selection pressure because of deleterious gene combination in homozygosis that can be responsible of vegetative growth problems and can hamper the germination and plantlet development (Dong et al., 2016; Lim and Earle, 2008). Then, the germination of embryos and the growth and development of the parthenogenetic plantlets is not always guaranteed. The mortality rate of embryos and plantlets *in vitro* is dramatically high, 30–85% (**TABLE 4.2**).

4.1.5.4. Chromosome Doubling

Plantlets that survive parthenogenesis are usually haploid (~70%), but mixoploid or even spontaneous DHs can be obtained too (Dong et al., 2016; Hooghvorst et al., 2020b). Spontaneous DH plantlets do not need to undergo chromosome doubling and can be directly acclimatized for DH seed recovery. On the other hand, haploid and usually mixoploid plantlets have to be chromosome doubled with antimitotic compounds. In cucurbit species, chromosome doubling has been reported chiefly in melon and cucumber. The doubling treatment can be *in vitro* or *in vivo*. The antimitotic compound can be colchicine, oryzalin or trifluralin. However, it is mostly performed using colchicine. Dinitroanilines,

oryzalin and trifluralin, have been reported as successful and very promising in cucumber (Ebrahimzadeh et al., 2018) and unsuccessful in melon 'Piel de Sapo' (Hooghvorst et al., 2020b). A high mortality might be recorded after the treatment, due to the toxicity of colchicine or the hyperhidricity suffered by the explants. On the other hand, *in vivo* treatment applies higher concentration of colchicine for a shorter time (2–3 h) by immersing the apical meristems of plants growing in the greenhouse into the colchicine solution. Chromosome doubling is highly influenced by the genotype and the most suitable method must be determined empirically. In melon, the efficiency of chromosome doubling in "Piel de Sapo" type genotypes was higher when applying colchicine at 5000 $mg \cdot L^{-1}$ for 2 h *in vivo* rather than at 500 $mg \cdot L^{-1}$ for 12 h *in vitro* (Hooghvorst et al., 2020b), or in a BC4F1 population, the 26% of haploids chromosome doubled when exposing shoot tip explants to 500 $mg \cdot L^{-1}$ for 3 h *in vitro* (Lim and Earle, 2009); in cucumber, colchicine was applied *in vitro* in solid E20H8 medium for 48 h or by submerging plantlet nodes and tips into a colchicine solution for 3–12 h reaching a 55% of chromosome doubling (Claveria et al., 2005) (**TABLE 4.2**).

TABLE 4.2. Summary of the parthenogenetic methodology and efficiency in cucurbit species for DH line production.

Species	Embryo detection method	Embryos per fruit	Mortality rate *in vitro*	Ploidy-level	Chromosome doubling		Reference
					Method	Efficiency	
Cucumber	X-ray	0.23	68.23%	62% H 38% M	E20H8 medium suppl. 500 µM colchicine for 48 h	55% M 30% DH	Claveria et al. (2005)
Cucumber	One-by-one	5.79	79.73%	-	-	-	Smiech et al. (2008)
Melon	One-by-one	6.27	30.85%	73% H 27% M	*In vitro* solution 500 mg·L^{-1} colchicine for 3 h	26% DH	Lim and Earle (2008)
Melon	X-ray	0.3	50.94%	73.08 H 23.08% DH 3.84% M	*In vivo* solution 5000 mg·L^{-1} colchicine for 2 h	9.38% M 20.31% DH	Hooghvorst et al. (2020)
Melon	One-by-one	1.97	34.22%	90% H	E20H8 medium suppl. 500 µM colchicine for 48 h	-	Gonzalo et al. (2011)
Melon	-	-	-	-	*In vivo* solution 0.5% colchicine for 2 h	46.03% DH	Solmaz et al. (2011)
Pumpkin	One-by-one	16.38	84.04%	5.90% H	-	-	Kurtar et al. (2009)
Pumpkin	One-by-one	69.85	-	0.86% H			Košmrlj et al. (2013)
Squash	One-by-one	0.2-10.5	71.20 - 80.10%	43.70% H 56.30% DH	-	-	Kurtar et al. (2002)
Squash	One-by-one	18.45	-	0.66% H			Baktemur et al. (2014)
Watermelon	One-by-one	1.4	-	-	-	-	Taşkın et al. 2013

Winter squash	One-by-one	13.72	85%	10.96% H	-	-	Kurtar and Balkaya (2010)

H haploid, M mixoploid, and DH doubled haploid.

4.1.5.5. DH Seed Recovery

Pure and viable seed must be recovered from chromosome doubled and the spontaneous DHs plants. Those plants present a low fruit set (~3-10%) when are self-crossed due to a low germination ability of pollen and the presence of different ploidy-levels in the whole plant (Lim and Earle, 2009). Abnormal ploidy-level in the same plant can be observed in the female and male flowers during the process. The self-crossing must be by hand-pollination to avoid external pollinations. In addition, the germination of DH seed is difficult and a generation for seed multiplication is usually required prior to F1 hybrid seed production.

4.2. GENOME EDITING IN CUCURBIT SPECIES

Genome editing techniques have the ability to introduce mutations in the plant genome. There are three main site-specific genome-editing nucleases with the capacity to target precise regions of the genome: zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats associated to nuclease Cas9 (CRISPR/Cas9). The CRISPR/Cas9 system has risen as the preeminent genome editing technique, due to its versatility, efficiency and ease to engineering in comparison to ZFN and TALENs. In cucurbits, there are no reports describing the application of ZNF nor TALENs, as far as the authors know. Nevertheless, CRISPR/Cas9 has been applied successfully in cucumber (Chandrasekaran et al., 2016; Hu et al., 2017), watermelon (Tian et al., 2017, 2018; Zhang et al., 2020) and melon (Hooghvorst et al., 2019a).

For the success of a CRISPR/Cas9 experiment, breeders need the sequenced genome of the target specie available, an adequate *Agrobacterium*-mediated transformation protocol and an efficient binary vector containing the sequence of the Cas9 protein capable to induce target mutations. In cucurbit species, the genomic sequences of cucumber, watermelon, melon, pumpkin, zucchini, squash, winter squash, bottle gourd and others are available at CuGenDB. Several CRISPR/Cas9 binary vectors have been reported successful in cucumber, watermelon and melon, and may be used for other cucurbits (**TABLE 4.3**). However, *Agrobacterium*-transformation protocol is still the main bottleneck to apply genome editing in cucurbits (Hooghvorst et al., 2019a; Zhang et al., 2020).

The production of genome-edited plants in cucurbit species usually starts with the selection of the gRNAs targeting the gene of interest and the construction of the CRISPR/Cas9 binary vector. The CRISPR/Cas9 vector containing the gRNAs should be tested prior to transformation in protoplast or *in silico* in order to corroborate the gene editing. The verification of the mutation induction before the *Agrobacterium*-mediated transformation process may ensure its success and reduce the time invested.

4.2.1. Agrobacterium-*Mediated Transformation*

The regeneration of transgenic plants in cucurbit species is considered a very recalcitrant process (Castelblanque et al., 2008; Hooghvorst et al., 2019a; Tian et al., 2017). In order to successfully obtain transgenic plants, regeneration and transformation have to take place. In cucurbits, cotyledonary explants are the main source of plant material for direct organogenesis, which is the production of adventitious buds or shoots from explants without a callus phase. Organogenesis is usually high in cucurbit species when using a suitable regenerating medium. Nevertheless, transformation, the acquisition of the transgene by the germinative cells, is highly inefficient. Therefore, during the process of *Agrobacterium*-mediated transformation, many plants regenerate but the vast majority lacks the transgene. The non-transformed regenerants (so-called "escapes") frequently grow into selective medium, contrary to what occurs in other species. The percentage of "escapes" can be ~30% and are a hindrance in order to select positive transformants. Moreover, the transformation process is usually optimized for and restricted to a few genotypes. This impedes the application of transformation and genome editing to a wide range of genotypes of interest. In comparison to the transformation efficiency of other species such as rice, Arabidopsis or tomato, cucurbit species have a low transformation of 1.32–5.63% (**TABLE 4.3**).

In addition, the ploidy-level of T0 generation can be spontaneously duplicated during the direct organogenesis resulting in tetraploid regenerants. Those polyploid plants are sterile and selfing is impossible for segregation of the transgene or for transgenic seed multiplication.

4.2.2. Genome Editing Efficiency

The mutation efficiency depends on the GC content of the sgRNA, the number of transformed cells and the Cas9 protein expression level in transgenic cells (Ren et al., 2019). In species such as rice or Arabidopsis, the efficiency frequently ranges between 80–100% and in cucurbit species it ranges from 20–100% (**TABLE 4.3**). Taking into account the larger effort invested in rice or Arabidopsis to apply and optimize CRISPR/Cas9 in comparison to cucurbit species, CRISPR/Cas9 efficiency in cucurbits can be

recognized as acceptable and suitable. Then, genome editing efficiency is not a bottleneck when applying CRISPR/Cas9 in cucurbits. Nevertheless, further attempts should be assayed, as done in other species, to achieve a higher genome editing efficiency such as using endogenous promoters for Cas9 and sgRNA expression, heat treatment during transformation, optimization of transformation efficiency (LeBlanc et al., 2018; Yan et al., 2015).

TABLE 4.3. Summary of the methodology and efficiencies of CRISPR/Cas9 reported experiments in cucurbit species.

Species	Target Gene	Plant Material	Transformation		Genome Editing Efficiency	Reference
			Method	Efficiency		
Cucumber	*eIF4E*	Cotyledonary explants	*Agrobacterium*-mediated	-	20%	Chandrasekaran et al. (2016)
Cucumber	*WIP1*	Cotyledonary explants	*Agrobacterium*-mediated	1.32%	65.2	Hu et al. (2017)
Watermelon	*PDS*	Cotyledonary explants	*Agrobacterium*-mediated	1.67%	100	Tian et al. (2017)
Watermelon	*PDS*	Protoplasts	Protoplast transfection	-	42.1-51.6%	Tian et al. (2017)
Watermelon	*ACS*	Cotyledonary explants	*Agrobacterium*-mediated	-	23%	Tian et al. (2018)
Watermelon	*PSK1*	Cotyledonary explants	-	2.30%	-	Zhang et al. (2020)
Melon	*PDS*	Cotyledonary explants	*Agrobacterium*-mediated	5.63%	42-45%	Hooghvorst et al. (2019a)
Melon	*PDS*	Protoplasts	Protoplast transfection	-	25%	Hooghvorst et al. (2019a)

4.2.3. Features to consider for further CRISPR/Cas9 experiments in cucurbits

Apart from *Agrobacterium*-mediated transformation, for a successful genome editing procedure there are few features to take into account such as the promoters used to drive the expression of the Cas9 and the gRNAs the potential off-targets of the CRISPR/Cas9 system, the heritability of mutations, and, the plant material.

4.2.3.1. Promoters

Trough *Agrobacterium* the transformation is achieved, and the plant acquire the transgene cassette carrying the Cas9 protein and the gRNAs. The transgene must be transcribed by the plant in order to express the endonuclease and edit the target region. The activity of the Cas9 is highly affected by its transcript levels. Cas9 gene transcription is usually driven by a strong constitutive promoter derived from a viral origin *CaMV 35S*. This promoter has been used to drive the Cas9 expression in many species, and it is the reference promoter to use. It has been widely used in dicots with mutation rates depending on the specie, ranging from 20% to 100% (Jiang et al., 2013; Pan et al., 2016). On the other hand, in monocots is less effective, and promoters such *ZmUBI* have been usually preferred (Christensen et al., 1992; Patro et al., 2012). In genome editing studies in cucurbits, *CaMV 35S* is the only promoter that has been used to drive the Cas9 expression. Nevertheless, several genes under the control of the same promotor or multiple incorporated copies of the same of transgene may lead to homology-dependent gene silencing and therefore impede genome mutation (Mette et al., 2000). The expression of sgRNA is usually driven by RNA polymerase III promoters, such as U3 or U6 (Belhaj et al.,, 2013). Those plant-origin promoters driving the sgRNA can be exchanged between species as long as they belong to the same class. For example, promoters from monocot species cannot be used for dicots, and vice versa. In cucurbits, U6 promoters from *Arabidopsis thaliana* have been used in cucumber, watermelon and melon, and a endogenous U6 promotor of cucumber was used by Chandrasekaran et al. (2016). The use of endogenous promoters driving the expression of sgRNAs seems to increase the transcription levels deriving in a higher mutation efficiency. In soybean, promoters from *Arabidopsis thaliana* and soybean were compared and

resulted in mutation efficiencies of 3.2-9.7% and 14.7-20.2%, respectively (Sun et al., 2015). Besides, depending on the aims of the CRSISPR/Cas9 study, tissue-specific promoters can be used to improve the heritability of induced target mutations and elucidate the functions of essential and pleiotropic genes (Ali et al., 2020). In cucumber, four *CsU6* promoters driving the expression of sgRNA were evaluated for its efficiency inducing mutations, and *CsU6-1* was selected for further experiments due to its higher mutation efficiency of 65.2% in comparison to *CsU6-2*, *CsU6-3* and *CsU6-4*, which resulted in 57.8%, 24.1% and 61.1%, respectively (Hu et al., 2017).

4.2.3.2. Temperature

The Cas9 protein homologue used for CRISPR/Cas9 system in plants is derived from *S. pyogenes*, a bacterium with an optimal growth temperature of 40ºC (Panos and Cohen, 1964). On the other hand, the *in vitro* plant material used in the CRISPR/Cas9 experiments is usually cultured at 22-24ºC. Therefore, the Cas9 protein is functioning at lower temperatures than its optimum, which can be detrimental for its activity (LeBlanc et al., 2018). An *in vitro* assay of the activity of SpCas9 at different temperatures showed a no reduction of its expression, but a decrease of 23% of cleavage in the target sites (LeBlanc et al., 2018). Heat shock stress has been applied to few species for now, Arabidopsis, Citrus, rice and maize. Arabidopsis transgenic *in vivo* T1 plants were exposed to heat stress treatment at 37ºC for 30h with a recovery of 42 h at 22ºC. The continuous growth at 37ºC was lethal. GFP reporter gene of transgenic plants was used as a target gene of the CRISPR/Cas9 system to better asses the mutation efficiency by analysing the GFP-fluorescence. Heat stressed plants showed a GFP-positive fluorescence of 12%, and control plants at 22ºC, 89%. (LeBlanc et al., 2018). In Citrus, *PDS* gene was targeted by CRISPR/Cas9 and *in vivo* plants were subjected to a cycle of 24h at 37ºC and a recovery of 24h at 24ºC for seven times. Plants exposed to heat shock stress showed an almost complete albino phenotype in the new developed tissues and plants grown at 24ºC did not show an enhancement of albino phenotype in young tissue (LeBlanc et al., 2018). Other researchers applied heat shock to rice protoplasts, which were subjected to different temperatures 22ºC, 28ºC, 32ºC and 37 ºC assaying Cas9 and three Cas12a nucleases. Protoplast transfection were comparable

between temperatures, and mutation frequency increased at 32ºC. Higher temperatures caused abnormality in cells, which were detrimental for cell viability or even lethal. For Cas12a nuclease, 28 ºC were shown as the optimal for its activity (Malzahn et al., 2019). Another CRISPR/Cas9 study in rice was performed to analyse the effect of heat-shock at calli and plantlet level. Fresh calli and one-week-old seedlings were subjected to 42ºC for 3h and then recovered at 25ºC for growth. Mutagenesis was analysed before and after the treatment, being 16% and 50-63.6% respectively (Nandy et al., 2019).

In cucurbit species, transformation process is highly limiting, and the addition of a heat-shock treatment could jeopardize the transformation efficiency due to sensitivity of plant material to high temperatures. Nevertheless, there is the need to adapt heat-shock treatment to optimize the best treatments, which in turn, could result in higher mutation rates and a low negative effect on plant growth. Fresh *in vitro* cotyledonary explants after one-week from *Agrobacterium*-mediated transformation could be a critic point where to apply it, because higher mutation rates at firsts steps of organogenesis could led to a higher number of mutation events of germinal cells. Approaches of heat-shock at *in vivo* plant level could be less aggressive for cell integrity.

4.2.3.3. Off-Targets

CRISPR/Cas9 undesired mutations caused by unspecific mutation of the system are called off-targets. The specificity of CRISPR/Cas9 is one of the most important features in comparison to other engineered nucleases. In plants, two strategies are usually followed to determine whether the system has caused undesirable off-targets, BLASTN or whole genome sequencing. The first one, uses a BLASTN algorithm to predict off-target sites which are similar to the target of interest and have the potential to being mutated according to the parameters of the algorithm. This approach is cheap and useful, but strong rules to predict off-targets are not already developed and available (Mao et al., 2017; Xie et al., 2014). In the second approach, whole genome sequencing, the genome of the CRISPR/Cas9 mutated plants is sequenced and compared with its WT for off-target evaluation. This approach is more expensive, but the results are more robust. In rice (Zhang et al., 2014), soybean (Sun et al., 2015), *Brassica Oleaceae* (Lawrenson

et al., 2015), and maize (Feng et al., 2016) a limited number of off-targets were identified applying whole genome sequencing. Nevertheless, if off-target mutations are identified, they could be removed by backcrossing. In four out of five reports in cucurbits, off-target analysis via BLASTN approach revealed no mutations in undesirable target sites. In Hooghvorst et al. (2019), potential off-target sites were searched and no off-target were detected with 0 to 2 mismatches as recommended by (Hahn and Nekrasov, 2019).

4.2.3.4. Heritability

The targeted mutations caused by CRISPR/Cas9 system in the T0 transformed plants are usually small deletions of one or two nucleotides in heterozygosis. Besides, other mutation events as larger deletions, insertions, substitutions or even inversions events can be present in the T0 plants in different edition profiles, such as heterozygous, if one allele remained WT; homozygous, if both alleles are mutated and present the same mutation; biallelic, if both alleles are mutated presenting different mutations; and, chimeric, if mutation analysis presents more mutation events than alleles, originated from different edited cell lines. The final objective of the CRISPR/Cas9 experiment is to produce plants with the gene of interest knocked-out due to a mutation event in homozygosity and evaluate the mutation effect for a concrete character. Because of the wide range of mutations events and edition profiles, T0 generation is usually segregated to obtain a T1, or even a T2, to stabilize the mutation events in homozygosity, avoiding chimerism and segregating out the CRISPR/Cas9 cassette transgene.

From the CRISPR/Cas9 cucurbit reports, Chandrasekaran et al. (2016) is the one that deeply analysed the heritability of mutations in cucumber. In his study, one (named CEC1-1) out of five transgenic T0 lines showed a heterozygous mono-allelic mutation event. The CEC1-1 T1 generation showed the same mutations as the T0. In another two transgenic lines (CEC1-1 and CEC2-5) mutation events were not detected in the T0 by PCR and restriction analysis. Nevertheless, its T1 and T2 generations showed a range of mutations and edition profiles, such as homozygous and heterozygous mutants and non-mutant plants. In another study in cucumber, the segregation of transgene in the T1 was 1:1,

suggesting a single copy insertion but no further analysis of mutation segregation events was described (Hu et al., 2017). Lastly, Tian et al. (2018) observations of mutations passed to the next T1 generation did not fit with the predictions of the Mendel's law of segregation, because of possible re-editing of target sequence. Other genome-editing studies in cucurbits did not analyse the segregation of mutation events because of the deleterious mutation of *PDS* gene (Hooghvorst et al., 2019a; Tian et al., 2017)

Many factors can affect the detection of the mutations in the T0 generation despite the presence of the Cas9 cassette and its expression such as low levels of expression and activity of the Cas9, different activity depending on the transgene insertion site, the expression of transgene in germ or somatic cells, a high percentage of faultless repairs after DSB or a high mutation frequency in the T1 generation due to the continuing activity of Cas9/sgRNA are commonly discussed as alternatives when heritability did not show an expected segregation pattern (Chandrasekaran et al., 2016; Howells et al., 2018; Mao et al., 2017; Tian et al., 2018).

4.2.3.5. Polyploidization of the regenerants

The expected ploidy of regenerants is expected to be diploid in cucurbit species, alike as the donor material, to be able to self-cross the T0 generation and obtain T1 seeds. Nevertheless, spontaneous duplication may occur during the *in vitro* process resulting in tetraploid regenerants. Polyploidization of cotyledonary cells due to an endoduplication has been reported (Guis et al., 2000). Shoots derived from leaf explants are less likely to present a polyploidization (20%) in comparison to those derived from cotyledons (70%) (Guis et al., 2000). The reduction of the co-culture period to one day decreases the number of tetraploid plants in comparison with higher durations. Therefore, flow cytometry or other determining ploidy-level techniques are frequently applied to screen diploid from tetraploid.

4.3. CRISPR/Cas9: A NEW ACTOR IN DH TECHNOLOGY

The haploid induction strategy is based on intraspecific crossing to obtain haploid progeny through an HI line. HI lines have the ability to produce *in vivo* haploid embryos upon pollination of a receptor line. Due to a mutation in a specific gene, which is essential for the normal fertilization of female gametic cells, fertilization is impeded, and egg cells develop into haploid embryos. Natural HI lines have been used since the beginning of modern breeding in maize (Coe, 1959), barley (Kasha and Kao, 1970), tobacco (Burk et al., 1979) and wheat (Laurie and Bennett, 1988). For instance, in maize, HI lines were discovered to carry a 4-bp insertion in the carboxy terminus of the MATRILINEAL (*MATL*) gene, also known as NOT LIKE DAD (*NLD*) (Gilles et al., 2017) or PHOSPHOLIPASE A1 (*PLA1*) (Liu et al., 2017), which encodes a pollen-specific phospholipase determined as the causal factor in the haploid induction process (Prigge et al., 2012). Phospholipase are essential enzymes expressed during pollen development and play a critical role in pollen development and germination and tube growth, its mutations are associated with delayed pollen germination and tube growth (Kim et al., 2011). Nevertheless, few species have natural HI lines; the production of HI lines in the laboratory through genome-editing techniques is a major challenge in haploid technology to improve and develop a new DH process of obtainment. As previously mentioned, TILLING, ZFNs and TALENs are potential genome-editing tools to produce positive mutants. Despite this, the random mutations of TILLING, and the complex, time-consuming engineering and unwanted off-target mutations of ZFNs and TALENs have meant that the CRISPR/Cas9 system has become genome-editing system of choice.

TABLE 4.4. Summary of the haploid inducer-mediated genome-editing systems using CRISPR/Cas9.

Species	Common name	Target gene	Haploid induction rate	Reference
Monocotyledonous				
Triticum aestivum	Wheat	*MATL*	18.9%	Liu et al. (2019)
Triticum aestivum	Wheat	*CENH3*	7%	Lv et al. (2020)
Oryza sativa	Rice	*MATL*	2-6%	Yao et al. (2018)
Zea mays	Maize	*DMP*	0.1-0.3%	Zhong et al. (2019)
Dicotyledonous				
Arabidopsis thaliana	Arabidopsis	*DMP*	2.1%	Zhong et al. (2020)
Arabidopsis thaliana	Arabidopsis	*CENH3*	1.1-44%	Kuppu et al. (2020)

DH technology has experimented a substantial progress resulting from the advances in engineered HI lines. Recently, *MATL* has become a target gene for genome editing in rice and maize with the CRISPR/Cas9 system, taking advantage of gene mutation during haploid induction (**TABLE 4.4**). In maize, maternal haploids were obtained with an efficiency of *ca.* 6.7% using the HI technique (Kelliher et al., 2017) and in rice, the average haploid induction rate was *ca.* ~6% (Yao et al., 2018). In wheat, a difficult species to work with because of their polyploidy, a 18.9% of haploid progeny was obtained using *MATL*-edited plants (Liu et al., 2019). On the other hand, another promising gene for HI, *DMP* (DOMAIN OF UNKNOWN FUNCTION 679 membrane protein), was demonstrated to induce haploids with a rate of 0.1%-0.3% in maize. *DMP* is a pollen-expressed gene highly expressed during the late stage of pollen

development and localized to the plasma membrane (Zhong et al., 2019). Despite of the lower rate of HI due to the loss-of-function of *DMP* gene, the combination of *ZmDMP* and *ZmPLA1* (or so-called *MATL* or *NLD*) genes knockout in the CAU6$^{(zmpla1\text{-}zmdmp)}$ line triggered and enhanced HI rate by 5 to 6-fold (Zhong et al., 2019) (**TABLE 4.4**). These rates of haploid production represent a breakthrough point that has the potential for further improvements. This new DH-generation pathway could be implemented for other secondary cereals like barley, oat, rye or triticale because existing DH protocols are less efficient than in species like rice, maize or wheat (Hooghvorst and Nogués, 2020). With many genetic transformation methods validated and whole genome sequencing available, breeders should be able to take advantage of haploid induction validated technology to increase the number of DHs and the efficiency of DH production in recalcitrant species via HI-mediated genome-editing technology, avoiding the need to test *in vitro* androgenic protocols for a range of genotypes.

Concerning dicotyledons, the main target for the HI technique has been the *CENH3* (centromeric histone 3) gene, which is a histone present in all plants that determines the position of the centromere and like other histones, it carries an N-terminal tail, which protrudes from the nucleosome and is a target for posttranslational modification and a C-terminal Histone Fold Domain, which interacts with DNA and other histones to form the nucleosome. *CENH3* plays a major role in chromosome segregation during mitosis, its alteration may have severe or even lethal consequences (Britt and Kuppu, 2016). Since its discovery, much research has been conducted to elucidate its function and ability during haploid induction in dicot species. Nevertheless, the specific mechanism and functions are still not clear. In 2010, major progress was achieved when studying the function of *CENH3* in *Arabidopsis thaliana* (Ravi and Chan, 2010). This work demonstrated that *GFP-tailswap* (*cenh3*-1 mutant plants) plants underwent haploid induction when crossed with a *CENH3* wild type line by eliminating chromosomes of the mutant line. Recently, the late HI related gene discovered was *DMP* was used for haploid induction in dicots. The loss-of-function of the *DMP* gene in Arabidopsis, a *ZmDMP*-like gene, induced haploid progeny in a rate of 2.1% (Zhong et al., 2020) (**TABLE 4.4**). Besides, in monocots the *CENH3* gene has been targeted too, it

was disrupted in maize through *CENH3-tailswap* transgenic complementation, reaching a 3.6% of HI (Kelliher et al., 2016), and more recently, in wheat, its knock-out reached a 7% of HI (Lv et al., 2020).

Hence, the *MATL, CENH3* and *DMP* genes are the current targets for HI-mediated genome-editing systems in monocots and dicots. However, other genes related to chromosome segregation during mitosis, or pollen development have potential as target genes for HI technology using CRISPR/Cas9, as reviewed by Ren et al. (2017).

Parthenogenetic approaches of haploid production via HI lines obtained by CRISPR/Cas9 can increase the chances of DH plant generation and ease the usual time-consuming and labour-intensive processes of androgenesis, gynogenesis and parthenogenesis (**FIGURE 4.2**). Further, the genotype dependency of many of the *in vitro* steps, like callus induction and plant regeneration, or *in vivo* steps like pollination with irradiated pollen, might be avoided using an HI approach, and this may also enable haploid plant material to be obtained from recalcitrant genotypes. To obtain *MATL*-, *CENH3*- or *DMP*-mutated lines for use as HIs, the transformation should be optimized in at least one genotype for each species because the regeneration and mutation processes are mandatory. This HI mutated genotype could be used to pollinate many genotypes of interest for haploid embryo generation, avoiding the need to optimize the process for recalcitrant genotypes.

The HI-mediated genome-editing approach for DH production for breeding purposes is a major discovery. Apart from improvements in the application of HIs discussed above, for a number of crop species the HI technique might be the best, if not the only way, to produce DHs. Much research needs to be done on this aspect to confirm the ease of work in parallel with better results. If the production of haploid plants is more efficient using the HI approach only the chromosome-doubling step at the haploid plant stage will become the bottleneck in achieving an efficient production of DH lines (**FIGURE 4.2**).

Another important approach to the HI technique is the HI-Edit system, where successful one-step genome-editing is achieved. Kelliher et al. (2019) crossed HI lines carrying the CRISPR/Cas9 cassette targeting genes for phenotypical evaluation with inbred lines to test the ability to produce positive genome-

edited mutants in the haploid offspring. The intraspecific crossing in maize led to a mutant haploid descendance of 2 to 8%, depending on the target gene. The interspecific crossing between a wheat inbred line and a maize line homozygous for the Cas9 gene, resulted in a 1.8% rate of mutant haploids. Moreover, in Arabidopsis, 17% of the offspring were mutant haploids when crossing a HI line with CRISPR/Cas9 system with an inbred line. Wang et al. (2019) applied the same system in maize and obtained 10 positive genome-edited plants among 245 haploids. This new system enables direct editing of elite inbred lines via a single crossing, thus overcoming recalcitrance.

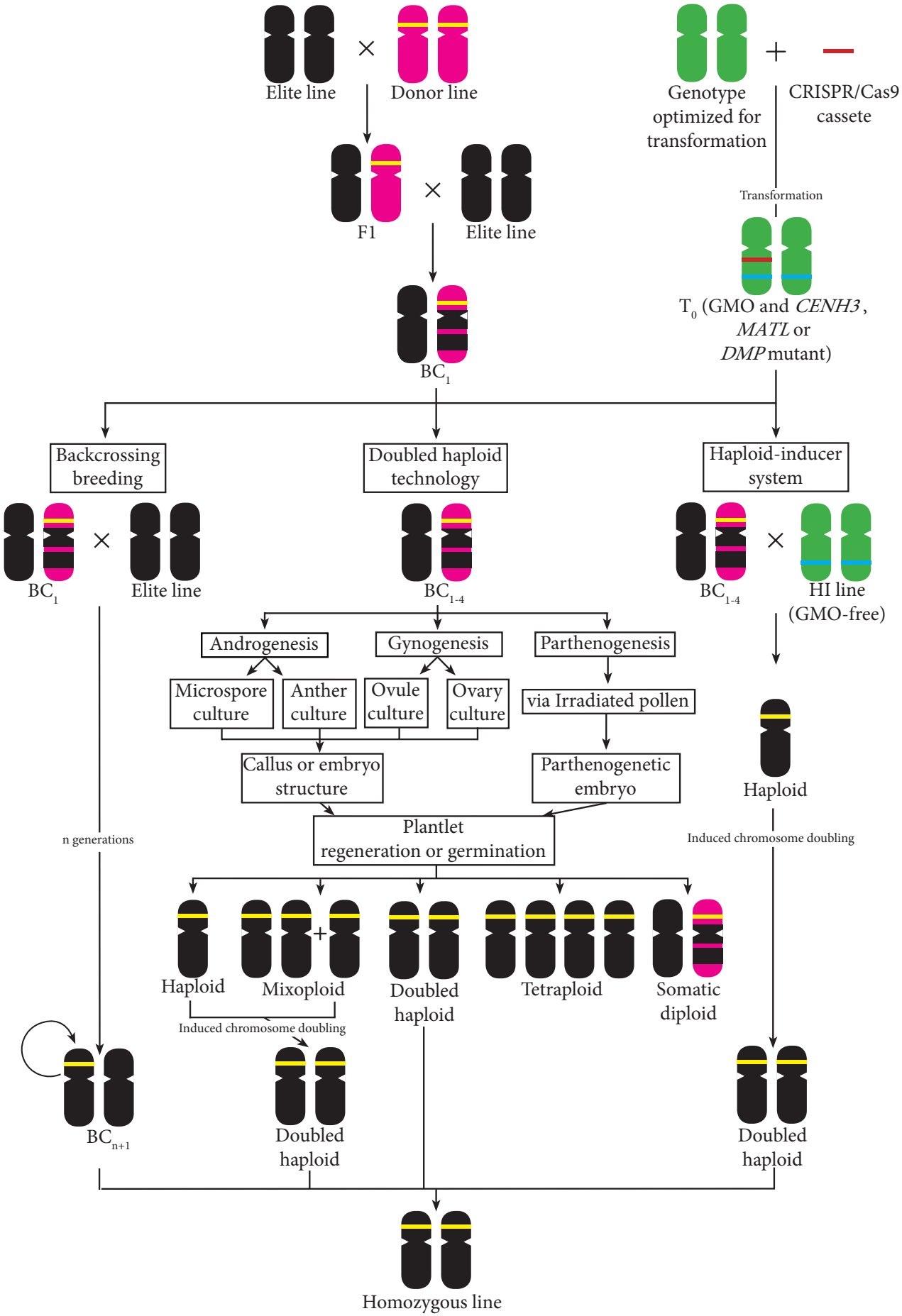
Elite line
Donor line
F1
Elite line
BC1
Genotype optimized for transformation
CRISPR/Cas9 cassete
Transformation
T0 (GMO and CENH3, MATL or DMP mutant)
Backcrossing breeding
Doubled haploid technology
Haploid-inducer system
BC1
Elite line
BC1-4
BC1-4
HI line (GMO-free)
Androgenesis
Gynogenesis
Parthenogenesis
Microspore culture
Anther culture
Ovule culture
Ovary culture
via Irradiated pollen
Callus or embryo structure
Parthenogenetic embryo
Haploid
n generations
Plantlet regeneration or germination
Induced chromosome doubling
Haploid
Mixoploid
Doubled haploid
Tetraploid
Somatic diploid
Induced chromosome doubling
BCn+1
Doubled haploid
Doubled haploid
Homozygous line

FIGURE 4.2. Schematic representation of three possible methods for producing purely homozygous lines: backcrossing breeding, doubled haploid technology and the HI-mediated CRISPR/Cas9 genome-editing system. Chromosome representation shows the genetic ploidy level according to number (one chromosome for haploids and two chromosomes for diploids) and the genetic background according to the color (black for elite receptor lines, pink for donor lines, green for genotypes optimized by genome transformation, yellow for the allele of interest, red for the CRISPR/Cas9 cassette targeting the *CENH3*, *MATL* or *DMP* gene and blue for the mutated *CENH3*, *MATL* or *DMP* gene). Backcrossing breeding can take from six to eight generations depending on the species and possible coupled to marker-assisted selection. DH technology can take from six months to two years depending on the species. An HI-mediated genome-editing system can take one year. DH technology and HI-mediated genome-editing system can start with BC_1 to BC_4 plants.

4.3.1. Next challenge: Haploid Inducer-Mediated Genome-Editing System in cucurbits

In plant breeding, important crops usually have a higher economical investment in order to increase their traits of interest. This is translated into a higher number of protocols and methodologies available for those species. DH technology follows the same pattern. However, sometimes, technical limitations related to a species or a genotype may rise difficulties to adapt a technique. It is the case of DH methodology in important families such as Cucurbitaceae, Solanaceae and Fabaceae, which despite of their economic worldwide importance, the progress achieved until nowadays is relatively low, in some cases.

An important family where HI-mediated genome-editing system could be advantageous is the Cucurbitaceae. Parthenogenesis via pollination with irradiated pollen is the best-known method to obtain haploid material among these species. However, the efficiency of the process is usually impeded by: (i) a high genotypic dependency; (ii) a low production of haploid embryos; and (iii) a difficulty to induce chromosome doubling of haploid plants due to mortality, hyperhidricity and a high ratio of haploids and mixoploid plants (Dong et al., 2016; Hooghvorst et al., 2020b). All this makes the process of DH production time-consuming and inefficient. The HI approach in the Cucurbitaceae would be similar to parthenogenesis via irradiated pollen due to the initial pollination of the receptor plant with the haploid-inducer pollen, and the rest of the process would be the same. Additional research is necessary to confirm whether the use of an HI line results in increased production of haploid embryos, reported rates lie

between 0 and 5% of seeds containing haploid embryos (Hooghvorst et al., 2020b). However, there are a few reports of the CRISPR/Cas9 genome-editing system being applied to cucumber, watermelon and melon with mutation efficiencies ranging from 42 to 100% (Chandrasekaran et al., 2016; Hu et al., 2017; Tian et al. 2017; Hooghvorst et al., 2019). On other important cucurbit species, such as *Cucurbita maxima*, *C. moschata* and *C. pepo*, there are no reports of successful generation of parthenogenetic DHs via irradiated pollen nor the application of CRISPR/Cas9 system yet. Nevertheless, HI-mediated genome-editing should afford great opportunities for breeding in these species as well. Therefore, the generation of haploid inducer lines in cucurbit species may be democratized targeting *CENH3* using CRISPR/Cas9, due to the specificity of CRISPR/Cas9 in multiple sequences of the *CENH3* gene which can be targeted efficiently. However, for the generation of haploid inducer lines mutated via CRISPR/Cas9 a successful transformation protocol would be imperative at least for one genotype. Generally, transformation protocol is adjusted for a specific genotype in cucurbit species. Fortunately, the genotype of the haploid inducer line is not an issue for haploid induction as long as it is the same species as the donor genotype from where to recover haploid embryos and bears male flowers. From the T0 CRISPR/Cas9 generation, heterozygous *CENH3*-mutated should be self-crossed in order to select: (i) T1 transgene-free and homozygous *CENH3*-mutated individuals, and (ii) transgene-free and heterozygous mutated-*CENH3* T1 individuals. The homozygous *CENH3*-mutated lines will be used for haploid induction process and heterozygous lines for haploid inducer line maintenance for future applications. Through this method, haploid inducer lines may be successfully maintained and used over the time (**FIGURE 4.3**).

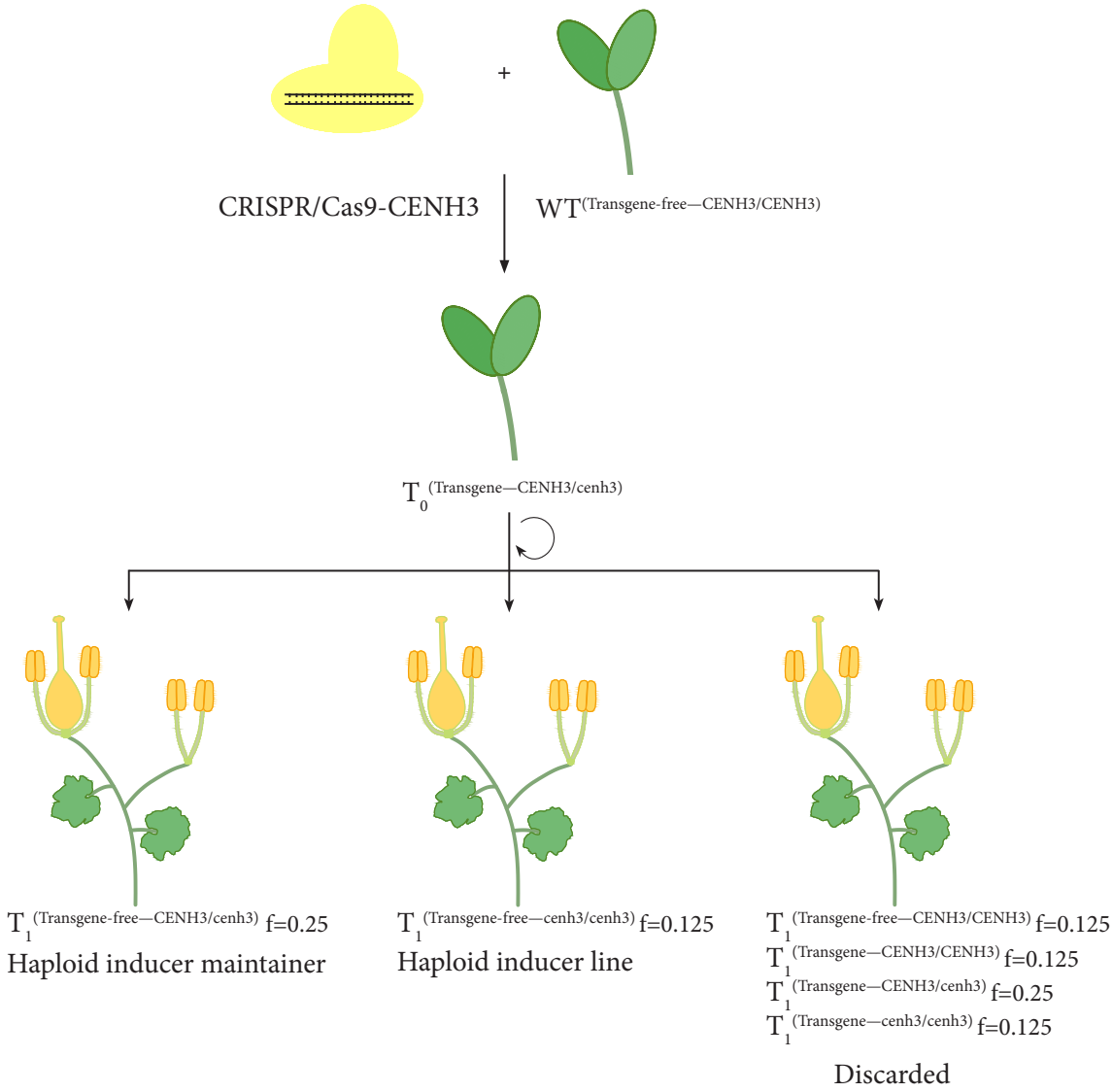

FIGURE 4.3. Schematic representation of the obtention of a haploid inducer line and its maintainer. WT: wild type.

The application of the haploid inducer-mediated genome-editing system in cucurbit species could reduce the actual time-consuming, labour-intensive and limited parthenogenic process. Although the process would remain very similar to parthenogenetic process with irradiated pollen due to the initial *in vivo* pollination with pollen of the haploid inducer line, the rate of haploid embryo induction could be significantly improved since no aggressive treatment, such as ionization, would be applied in haploid inducer line pollen. Furthermore, no irradiation source equipment will be required as it is not always available in all laboratories. On the other hand, parthenogenesis via irradiated pollen is highly genotype-

dependent and accomplishment of irradiation depends upon the radiation resistance of the pollen. In addition, some genotypes are reported to be very recalcitrant to induction of haploid generations when pollinated with irradiated pollen. Pollination with pollen of the haploid inducer lines could broaden the range of genotypes that can produce haploids and increase the number of parthenogenetic embryos. This must be assayed to see whether haploid inducer lines can increase the number of haploid embryos produced in cucurbits.

Moreover, parthenogenesis with irradiated pollen is routinely applied in cucumber and melon and substantial progress has been made. In contrast, less effort has been applied in species such as watermelon, winter squash, pumpkin or bottle gourd and, therefore, less progress has been made in optimizing their parthenogenetic protocols. In cucurbit species, the fruits set once pollinated with irradiated pollen or pollen from haploid inducer lines will follow the same steps as in *in situ* parthenogenesis via irradiated pollen. Consequently, limiting factors described for parthenogenesis with irradiated pollen would likewise be present using the haploid inducer approach. Therefore, the haploid inducer-mediated genome-editing approach can take advantage of the progress made to successfully obtain doubled haploid lines and increase the current state of *in situ* parthenogenesis.

The haploid inducer-mediated genome-editing approach is an opportunity to improve the efficiency of doubled haploid production in recalcitrant species. The use of *matl* or *cenh3* mutant lines in cereals or dicots for haploid induction and production might avoid androgenesis, gynogenesis or parthenogenesis via pollen irradiated and boost their obtention in several species. Besides, HI-Edit system could boost the production of haploid plants mutated in one generation, these could ease the work of obtaining new cultivars.

4.3.2. Other challenging families: Solanaceae and Fabaceae

It is worth noting that DH production in crop species like those from the Solanaceae could be greatly improved thanks to the HI technique too. Solanaceae species are very recalcitrant to *in vitro* DH

processes and a methodology has not been established yet for a number of species, making it difficult for breeders to use DH technology on a routine basis, as far as we know (Seguí-Simarro et al., 2011). For instance, DH lines can be obtained efficiently in eggplant and bell pepper through anther culture (**TABLE 4.1**). Nevertheless, to our knowledge there is currently no suitable DH method available for tomato, despite all the efforts invested in DH production in this major horticultural crop. Thus, classical breeding is the only method to obtain new commercial tomato cultivars, complicating the advances of breeding selection. Therefore, generation of a HI *CENH3*-mutant tomato line via genome-editing could represent a breakthrough. This could lead to a new era for tomato breeding, avoiding the current generations of self-pollination that are still required to produce inbred lines for use in hybridization. Fortunately, delivery of CRISPR/Cas9 by *Agrobacterium*-mediated transformation has been reported several times in tomato (Van Eck, 2018). CRISPR/Cas9 has been successfully applied in tomato with mutation efficiencies of 80-100% for applications such as studying mutation stability of heredity in later generations (Pan et al., 2016), obtaining parthenocarpic tomato fruits (Ueta et al., 2017), and increasing plant resistance to powdery mildew (Nekrasov et al., 2017).

In the Fabaceae, attempts to produce DHs have been reported in many species (soybean, field pea, chickpea, peanut and common bean), mainly via androgenesis. Leguminous species are particularly important for low input and sustainable cropping due to their ability to fix nitrogen and as a dietary protein source for human food and animal feed (Croser et al., 2007). Nonetheless, not much progress has been made with DH technology applications because there has been little research undertaken on these species, and the induction and regeneration rates are inherently low. Some DH lines have been produced thanks to spontaneous doubling and high rates of somatic regeneration (Croser et al., 2007; Ochatt et al., 2009). However, successful induction of chromosome doubling has not been widely reported in this family because of the scarcity of the obtained haploid material. Significantly, the CRISPR/Cas9 system has been applied in soybean multiple times (Chilcoat et al., 2017; Sun et al., 2015) and should be extended to other Fabaceae species. The HI-mediated CRISPR/Cas9 genome-editing technique presents a great opportunity to produce DHs in these species.

Despite all the other improvements in DH line production that have emerged, chromosome doubling is one step that has been inherited from the classical DH approach (**FIGURE 4.2**). It is therefore of major importance to adapt and optimize new chromosome-doubling protocols via antimitotic compounds to increase the number of DH lines derived from the improved HI protocols. Some species, such as sorghum and bell pepper, do not have an optimized well-described chromosome-doubling protocol because of their high level of spontaneous DH regenerants (**TABLE 4.1**). For instance, in rice, the spontaneous chromosome-doubling rate is usually very high, ranging from 30 to 80% (**TABLE 4.1**), and induced chromosome doubling has been ignored since the generation of the first DHs (Niizeki and Oono, 1968). Indeed, before 2019 no reliable reports on doubling rice plants were published, authors treated androgenic haploid plants with a recovery of 35% (Hooghvorst et al., 2020a). On the other hand, species whose DH production is impeded by poor performance in tissue culture do not have a reliable described method for chromosome doubling; this is the case of rye, watermelon, other secondary cucurbit species, tomato and leguminous species (Croser et al., 2007; Dong et al., 2016; Forster et al., 2007; Seguí-Simarro et al., 2011).

4.3.3. HI-CRISPR/Cas9 legislative future

Another important aspect of HI-CRISPR/Cas9 based technology is the legislation put in place to regulate the development and commercialization of genetically modified organisms (GMOs) in the EU, and which handles issues of uncertainty and safety (Sprink et al., 2016). The Directive 2001/18/EC defines a GMO as an organism "in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination", and GM techniques are further described as "recombinant nucleic acid techniques involving the formation of new combinations of genetic material". In our opinion, haploid production through CRISPR/Cas9 mutants used for HI needs to be exempt from this regulation because the resultant GMO-free haploid line would never have recombinant DNA incorporated into its single progenitor cell.

The US regulatory framework would categorize HI-CRISPR/Cas9 as a null segregants because transgenic sequences are eliminated from the final product. However, this technique has the precedent of centromere-mediated chromosome elimination (CCE) through a *GFP-tailswap* transgenic line for which the Plant Health Inspection Service (APHIS) determined that the progeny obtained from CCE would not be subjected to its regulation (Camacho et al., 2014).

Nevertheless, as pointed out by Abbott (2015) and Camacho et al. (2014), the EU and the US criterion in the current legislation is based on the process rather than the product. Therefore, a process that uses recombinant technology could create controversy for regulatory institutions, despite the impossibility of detecting the haploid origin. The regulatory framework should be based on science and able to evolve together with the scientific knowledge and technologies.

4.4. FINAL REMARKS

Plant breeding is a set of methods and techniques used in science to obtain plants with the desired traits beneficial for human or animal consumption. Since the early days of agriculture and the domestication of wild species into crops until nowadays, plant breeding has experienced a great technification with a constant increase of techniques, approaches and methods which enables to change, improve or delete a trait, allowing a great control on plant breeding.

In this thesis, two major techniques, DHs and CRISPR/Cas9, have been studied and applied in a relatively important crop such as melon. DH technique in melon has been largely developed during the past two decades; however, it is still a laborious procedure and might be eased using new approaches. In our DH study, we applied the technique in order to obtain commercial cultivars at the same time that we reported a parthenogenetic study in melon 'Piel de Sapo' genotypes, which are highly recalcitrant. The CRISPR/Cas9 technique is currently being applied in several laboratories, as far as the author knows. However, the targeting of *CmPDS* gene is the first published report of CRISPR/Cas9 in this species. This study attempts to serve as a first step in the path of obtaining a highly optimized protocol for melon, therefore, its value relies on trying to boost the firsts steps of the process. In this sense, further research is yet to be published and developed. Over the coming years, CRISPR/Cas9 will undergo a great improvement thanks to the work of the past few years.

Thereafter, the next logical step is to combine both techniques (DH and CRISPR/Cas9) in order to produce haploid inducer lines used for DH production. This milestone could switch the paradigm of the DH process in cucurbits and melon to results highly efficient. Although there is still work to do and it is not sure that HI lines present a higher haploid induction in comparison to the current *in situ* parthenogenesis with irradiated pollen, it is worth the effort. Moreover, the development of HI lines will open the possibility to apply HI-Edit, a technique that could allow the production of haploids with targeted mutations, a very useful application to produce commercial cultivars in a reduced time.

Cucurbit species suffer a delay in the development and optimization of breeding techniques, such as genome editing, and this could result in a huge distance in comparison with more important species in the state-of-the-art breeding technologies that will exponentially be developed in the further years. In addition, genome editing may suffer a constriction too, as it may be stigmatized (if it is not yet) and dropped due to social concerns over health issues, as GMO techniques were.

Therefore, breeders that attempt to optimize and develop breeding techniques directly or indirectly used to produce new cultivars should be able to apply every tool in the breeder's toolbox, as long as their health and environmental safety have been scientifically proved. To sum up, literally quoting Breseghello and Coelho (2013), "in industrialized countries, only a small portion of the population is engaged in agriculture. The vast majority of people rely on a tacit social pact for their survival, which assures that someone will provide food in exchange for some service or good. This pact is so basic to modern life that people take for granted that food is available in the nearest supermarket. However, agriculture failure could cause a disruption of this pact, leaving people in a situation of food insecurity. Thus, protecting agriculture means warranting the foundation pact of modern civilization".

5. CONCLUSIONS

- The *in situ* parthenogenetic capacity of "Piel de Sapo" germplasm was evaluated, showing: a low capacity of fruit set when pollinated with irradiated pollen, a low production of parthenogenetic embryos, a poor *in vitro* culture performance, a low chromosome doubling and a low fruit set of DH lines once chromosomes were doubled.

- The "Piel de Sapo" *inodorus* type can be considered as a recalcitrant genotype for parthenogenesis in melon species in comparison to other genotypes. Nevertheless, we succeeded in obtaining DH seeds that have a great value for hybrid F1 seed production and commercialization.

- During the parthenogenetic process, the X-ray method was used as the most successful and optimum method to detect and rescue parthenogenetic embryos. The poor performance of "Piel de Sapo" genotypes showed during *in vitro* culture could be enhanced by changing media composition instead of using the traditional E20A medium.

- An *in vivo* chromosome doubling method with colchicine resulted as the most successful methodfor chromosome doubling of haploid plants, in front of *in vitro* chromosome doubling methods with colchicine or oryzalin.

- Efficient gene editing in melon presents the possibility to study new gene functions for basic research, and new opportunities for melon productivity by improving biotic stress, melon production and the post-harvest utilization. Towards this, we demonstrated, for the first time, the occurrence of targeted mutagenesis with the CRISPR/Cas9 system in melon protoplasts and plants.

- The described *CmPDS* knockout system described generates easily detectable albino and dwarf plants and mutation events in a frame of 6–8 weeks. Therefore, it provides a valuable

method to facilitate rapid assessment and optimization of CRISPR/Cas9 and other genome-editing technologies in melon.

- We have drawn general conclusions about the success of different approaches to DH generation and the implications of the existing technologies of DH production and chromosome doubling for future research.

- DH technology has been a major boost for plant breeding, reducing the time and labour required to derive new breeding varieties. Among the DH processes, chromosome doubling is often overlooked due to the importance of the haploid induction step or the high frequency of spontaneous DHs, and this is reflected in the absence of DH protocols or a lack of efficiency in those that exist.

- Androgenesis via anther culture is the most common protocol for haploid and DH plant production, being the predominant method in vegetable and horticultural crops, and the only method used in cereals.

- The chromosome-doubling step is far from being settled and there is a need to continue investigating new protocols based on new or existing antimitotic compounds to reduce toxicity-related mortality and to attain higher frequencies of chromosome doubling.

- Induced chromosome doubling has a genotypic dependency, and even species with a high rate of spontaneous doubling should not be ignored when developing efficient chromosome-doubling methods because some genotypes are unable to regenerate spontaneously.

- The only adaption required to the tissue culture technique will be the regeneration of CRISPR/Cas9 genome-edited plants, which will be a much easier task because only one genotype will be sufficient as the pollen donor for haploid induction across a diversity of receptor genotypes of the same species.

- The uprising CRISPR/Cas9-based gene targeting approach for haploid induction will make the chromosome-doubling step inexorable, because of the low or absent spontaneous chromosome doubling in haploid induction process. Therefore, new *in vitro* or *in vivo* chromosome-doubling protocols will be needed for species where haploid induction has not yet been yet reported due to low regeneration efficiency or a complete lack of regeneration success.

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ANNEX I – PUBLISHED ARTICLES

CHAPTER 1. *In situ* parthenogenetic doubled haploid production in melon 'Piel de Sapo' for breeding purposes

frontiers in Plant Science

ORIGINAL RESEARCH
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In situ Parthenogenetic Doubled Haploid Production in Melon "Piel de Sapo" for Breeding Purposes

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Doubled haploids in cucurbit species are produced through *in situ* parthenogenesis via pollination with irradiated pollen for further use as parental lines for hybrid F1 production. In this study, seven genotypes of melon "Piel de Sapo" were appraised for agronomic traits and pathogen resistances to evaluate its commercial value and used as donor plant material for the parthenogenetic process. Then, *in situ* parthenogenetic capacity of melon "Piel de Sapo" germplasm was evaluated and optimized. Several steps of the parthenogenetic process were assessed in this study such as melon fruit set after pollination with irradiated pollen, haploid embryo obtention, *in vitro* germination and growth of parthenogenetic embryos and plantlets, *in vitro* and *in vivo* chromosome doubling with colchicine or oryzalin and fruit set of doubled haploid lines. Parthenogenetic efficiencies of "Piel de Sapo" genotypes showed a high genotypic dependency during the whole process. Three different methods were assayed for parthenogenetic embryo detection: one-by-one, X-ray and liquid medium. X-ray radiography of seeds was four times faster than one-by-one method and jeopardized eight times less parthenogenetic embryo obtention than liquid medium. One third of melon fruits set after pollination with irradiated pollen contained at least one parthenogenetic embryo. The 50.94% of the embryos rescued did not develop into plantlets because failed to germinate or plantlet died at the first stages of development because of deleterious gene combination in haploid homozygosity. The distribution of the ploidy-level of the 26 parthenogenetic plantlets obtained was: 73.08% haploid, 23.08% spontaneous doubled haploid and 3.84% mixoploid. Two *in vitro* chromosome doubling methods, with colchicine or oryzalin, were compared with a third *in vivo* colchicine method. *In vivo* immersion of apical meristems in a colchicine solution for 2 h showed the highest results of plant survival, 57.33%, and chromosome doubling, 9.30% mixoploids and 20.93% doubled haploids. Fruit set and seed recovery of doubled haploids lines was achieved. In this study, doubled haploid lines were produced from seven donor genotypes of melon "Piel de Sapo," however, further improvements are need in order to increase the parthenogenetic efficiency.

Keywords: melon, parthenogenesis, doubled haploid, chromosome doubling, "Piel de Sapo," X-ray, colchicine

INTRODUCTION

Melon (*Cucumis melo*) is a eudicot diploid plant species from *Cucurbitaceae*. Melon has been divided in two subspecies, subsp. *melo* and subsp. *agrestis*, and 19 groups have been described by Pitrat (2016): *acidulus*, *agrestis*, *ameri*, *cantalupensis*, *chandalak*, *chate*, *chinensis*, *chito*, *conomon*, *cassaba*, *dudaim*, *flexuosus*, *ibericus*, *inodorus*, *indicus*, *kachri*, *makuwa*, *momordica*, and *tibish*. Together with cucumber (*Cucumis sativa*) and watermelon (*Citrullus lanatus*), melon is one of the most economic important species from *Cucurbitaceae*. Melon production was about 32 million tons in 2017 (FAO, 2017), being China, Turkey, Iran, Egypt, India, Kazakhstan, United States, and Spain, the major producers ordered according to its yield. The melon fruit has a huge genotypic diversity and each country has its own preferences due to cultural reasons (Monforte et al., 2014). *Inodorus* and *Cantalupensis* are the most produced melon groups in Spain. Pathogens are a major threat to melon productivity, Zitter et al. (1996) estimated that over 200 pathogens affected the productivity of cucurbits, caused by fungi, bacteria, viruses or mycoplasma organisms. It is estimated that diseases can cause yield losses of more than 30–50% in melon cultivation (El-Naggar et al., 2012). Powdery mildew, fusarium wilt, and melon necrotic spot virus (MNSV) are the most critical diseases in melon and cucurbit species. Consequently to the high impact of pathogens in cucurbits many modern breeding programs have been implemented to obtain resistant cultivars (Kuzuya et al., 2003; Lotfi et al., 2003).

Commercial seed of melon cultivars can be open pollination (OP) or hybrid F1 cultivar (Robinson, 2000). OP cultivars are inbred lines obtained through several rounds of self-crossing until the obtention of a high homozygous and stable line. On the other hand, hybrid F1 cultivars are stable but heterozygous lines obtained from the cross of two homozygous lines. Hybrids F1 take advantage of heterosis for major fruit yield and pathogen resistances and have a great importance in the European market in spite of its production costs (McCreight et al., 1993; Robinson, 2000). Hybrids F1 are produced by crossing two pure parental lines, which can be obtained by successive rounds of self-crossing and selection during classical breeding or by biotechnology approaches, like doubled haploids (Dong et al., 2016). Doubled haploids (DHs) are pure homozygous lines which require shorter time to produce in comparison to classical breeding (Germanà, 2011). DH lines are generated by androgenesis, gynogenesis or parthenogenesis in major crops, and can be used as a parental for hybrid F1 production or as a stable line. In cucurbit species, *in situ* parthenogenesis through irradiated pollen is the most common and efficient method to obtain haploid plants (Sauton and Dumas de Vaulx, 1987). Those haploid plants need to undergo chromosome doubling using antimitotic compounds. *In situ* parthenogenesis in cucurbits to produce haploid embryos is usually low, from 0 to 5% of seeds contain haploid embryos (Dong et al., 2016), and is less efficient and more time-consuming than other crop species such as: wheat (Niu et al., 2014), bell pepper (Irikova et al., 2011), rice (Hooghvorst et al., 2018), or onion (Fayos et al., 2015).

First haploids of melon, embryos and plants, were obtained by an interspecific crossing with *Cucumis ficifolius* (Dumas de Vaulx, 1979). Then, *in situ* production of haploid embryos was achieved thought pollination with irradiated pollen (Sauton and Dumas de Vaulx, 1987). The pollination of a female flower stigma with irradiated pollen stimulates an *in situ* parthenogenetic response when pollen tube reaches the egg-cell. Then, parthenogenetic haploid embryo is developed, extracted and cultured *in vitro*. Germinated embryo regenerates into a full-developed plantlet that need to undergo chromosome duplication for DH seed recovery. Nevertheless, *in situ* parthenogenesis in cucurbits and specifically, in melon, has many bottlenecks that reduces its efficiency in each step of the process. Melon parthenogenesis has a high genotypic dependency and methodological issues that impede the efficient production of DHs such as: low levels of female flowers developed once pollinated with irradiated pollen; low production of haploid embryos; difficulty to detect seeds containing haploid embryos; low germination of haploid embryos *in vitro*; high mortality of germinated embryos and growing plantlets; very low or null spontaneous chromosome duplication; difficulty to induce chromosome doubling in haploid plants due to a high mortality and hyperhidricity; high ratio of haploid and mixoploid plants; low pollen germination levels of chromosome doubled plants which trigger a decrease of fruit set and seed recovery; and, low DH seed germination (Lim and Earle, 2008, 2009; Gonzalo et al., 2011; Dong et al., 2016).

The seeds of melon fruits set produced via pollination with irradiated pollen are inspected in search of parthenogenetic embryos. The inspection of seeds one-by-one under a stereo microscope is successful and the most commonly applied although its time-consuming and labor-intensive. Two other methods have been reported for parthenogenetic haploid embryo detection such as X-rays, which had been proven efficient but demand high equipment specialization, and liquid culture, which had been proven ineffective (Dong et al., 2016). The low rate of spontaneous chromosome doubling during melon parthenogenesis process require the implementation of a chromosome doubling step using antimitotic compounds. Colchicine has been the most used antimitotic in melon for chromosome doubling, either via immersion of *in vitro* shoot tips or nodular explants, or via immersion of *in vivo* shoot tips. Chromosome doubling rate can range from 0 to 90% depending on the genotype (Yetisir and Sari, 2003; Lim and Earle, 2008; Gonzalo et al., 2011; Solmaz et al., 2011).

The main objective of this study was to evaluate the commercial value and the parthenogenetic capacity of seven genotypes of *C. melo* var. *Inodorus* "Piel de Sapo" type to obtain DH lines which might be further used as parental lines for commercial hybrid F1 seed production. Moreover, the parthenogenetic generation of DHs from the seven genotypes was evaluated and optimized through the analysis and description of the different steps of the process, assaying: three haploid embryo rescue protocols, previously described in the literature; three chromosome doubling methods; and, a new cytometry flow method for evaluating the ploidy-level.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seven genotypes of *C. melo* subsp. *melo* "Piel de Sapo" *indodorus* type were used as plant material (provided by ROCALBA S.A.). Six genotypes were inbred lines (PS-1305, PS-1901, PS-0301, PS-0709, PS-2001, and PS-2301) and one genotype was an open pollinated cultivar (Melito). Melon plants were grown in greenhouse conditions at *Servei de Camps Experimentals* at the *Universitat de Barcelona* (Barcelona, Spain) in 9 L plastic containers filled with substrate containing Floratorf peat moss (Floragard Vertriebs, Oldenburg)—vermiculite (2:1 v/v) substrate supplemented with Osmocote (The Scotts Company LLC, United States) and 1 g $CaCO_3$ per peat liter was added to adjust the substrate pH to 6 (**Figure 1A**).

Pollination With Irradiated Pollen, Parthenogenetic Embryo Rescue, and Germination

Male flowers containing mature pollen were collected early in the morning and irradiated at 250 Gy using a ^{137}Cs source at *Centres Científics i Tecnològics* at the *Universitat de Barcelona* (Barcelona, Spain). Female flowers were emasculated, pollinated with the help of a brush, and bagged to avoid external pollinations. Each female flower receptor was pollinated with three to five irradiated male flowers (**Figure 1B**). Pollination was done at the same and the next day of irradiation. After 3 weeks, melon fruits that set were harvested and opened for seed collection. Three different methods of seed inspection and embryo detection and rescue were assayed: seed inspection one-by-one with the help of a light box, X-ray radiography of seeds and floating seeds in liquid medium. In the one-by-one individual inspection of seeds method, seeds were sterilized in 20% sodium hypochlorite supplemented with 4 drops·L^{-1} of Tween 20, rinsed three times in sterile water and opened one-by-one with the help of a stereo binocular microscope and a light box. In the detection of embryos by X-ray radiography method, seeds were placed in an acetate sheet (**Figure 1E**) on the Imaging Screen K, introduced in a Faxitron® cabinet X-Ray system (Hewlett Packard, Palo Alto, CA, United States) to be exposed to 16 kV during 70 s and the seeds containing embryo were selected and sterilized (**Figure 1F**). For liquid culture of seeds, seeds were sterilized and cultured *in vitro* in E20A liquid medium in jars. Detected seeds containing embryos with the three methods were manually opened in aseptically conditions and embryos were cultured *in vitro* in E20A solid medium (Sauton and Dumas de Vaulx, 1987) in 90 mm petri dishes.

Chromosome Doubling

Three different protocols were assayed to induce chromosome doubling in haploid plantlets, two *in vitro* and one *in vivo*. The two *in vitro* treatments used nodes and shoot tips as plant material, the protocol of Lim and Earle (2009) applied 500 mg L^{-1} of colchicine for 12 h; and, the protocol of Ebrahimzadeh et al. (2018) applied 50 mg L^{-1} of oryzalin for 18 h. Each chromosome doubling method was performed three to five times in different days to treat them as independent replicates. For both *in vitro* treatments, *in vitro* haploid plantlets were micropropagated aseptically, nodes with two to three axillary buds and shoot tips with one to two axillary buds were treated in E20A liquid medium supplemented with the antimitotic solution and DMSO 2% (v/v) during the correspondent exposition time. Once the expositition time elapsed, nodes and shoot tips were rinsed with water and cultured in E20A solid medium. The third chromosome doubling protocol assayed was *in vivo*, following the methodology of Solmaz et al. (2011) and Yetisir and Sari (2003). *In vitro* haploid plantlets were acclimated in the greenhouse (**Figure 1J**) and when plants expanded four to eight leaves, apical stem was submerged in a 5,000 mg L^{-1} colchicine solution supplemented with 2 drops·L^{-1} of Tween 20 for 2 h (**Figure 1K**). Apical stems were rinsed with water after the treatment.

When apical stems expanded new leaves since the application of the antimitotic treatment, ploidy-level was determined to identify the induced chromosome doubled plants. *In vitro* chromosome doubled plantlets that survived and grew roots were acclimatized in a growth chamber at 25°C, illuminated with 50–70 μmol m^{-2} s^{-1} fluorescent light under a 16/8 h day/night photoperiod and covered with plastic pots. After 2 weeks, the plantlets that survived were acclimatized in the greenhouse (**Figure 1J**). The stems of *in vivo* treated plants that remained haploid were pruned, and the ones that chromosome doubled were grown until flowering and autopollinated. If plants showed phenotypical mixoploidy, carrying male flowers with and without pollen, stems with haploid flowers were pruned.

Ploidy-Level Determination

The ploidy-level of germinated parthenogenetic embryos and antimitotic treated plants was determined by flow cytometry following the procedure of Hooghvorst et al. (2018) with slight modifications. About 5 mg of young leaves were collected and put into ice-cold 2 mL microcentrifuge tubes each with a steel bead (3 mm diameter). To each tube, 300 μL of cold lysis buffer (0.1 M citric acid and 0.5% Triton X-100 in distilled water) were added. Tubes were cooled at −20°C for 10 min. Samples were shaken at 25 Hz for a total of 20 s in a MM 400 tissue lyser (Retsch, Haan, Germany) two times and tubes were vortexed with a vortex mixer between the two triturations. The aliquot from each tube was filtered through a 22 μm nylon filter (Sefar Maissa, Blacktown, Australia), gently vacuumed and transferred to a flow cytometry sample tube (Beckman Coulter Inc., Pasadena, CA, United States). Afterward, 150 μL of propidium iodide (PI) stain solution [0.25 mM Na_2HPO_4, 10 mL 10× stock (100 mM sodium citrate, 250 mM sodium sulphate) and 9 M PI made up to 100 mL with Milli-Q water] was added to each tube. Tubes were then sealed and kept on ice in the dark for 1 h before flow cytometry analysis. The stained nuclei samples were analyzed using a GalliosTM Flow Cytometer (Beckman Coulter Inc., Pasadena, CA, United States) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centers, University of Barcelona) and a 32-well carrousel. One diploid control sample was included every seven measurements. Flow cytometry data was analyzed using Summit Software v4.3 (Beckman Coulter Inc., Pasadena, CA, United States).

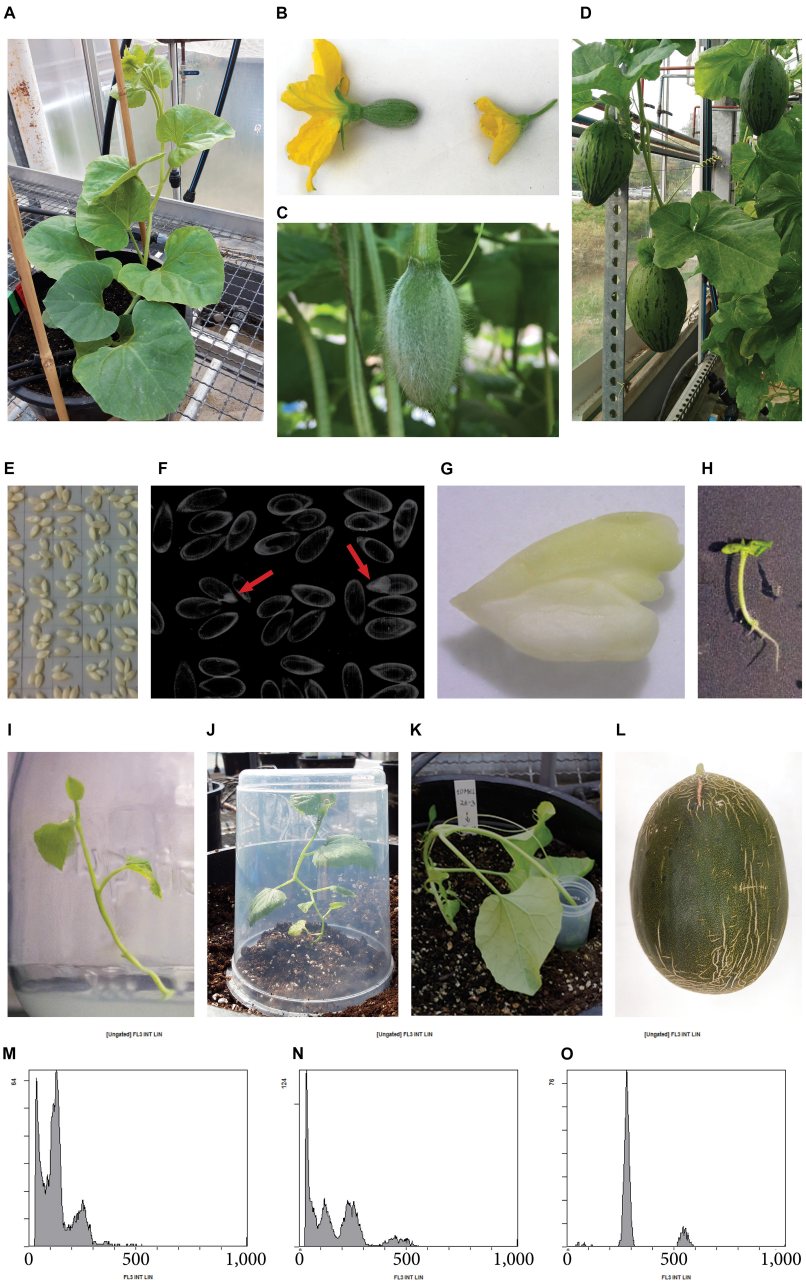

FIGURE 1 | Production of parthenogenetic DH lines in melon "Piel de Sapo" genotype. **(A)** Melon donor plant. **(B)** Detail of a female flower **(left)** and a male flower **(right)**. **(C)** Female flower developed after pollination with irradiated pollen. **(D)** Melon fruits set after 3 weeks of pollination with irradiated pollen, which potentially contain parthenogenetic embryos in their seeds. **(E)** Melon seeds in an acetate sheet ready to be photographed by X-ray. **(F)** X-ray photography of seeds, parthenogenetic embryos are pointed with a red arrow. **(G)** Parthenogenetic embryo rescued. **(H)** Germinated embryo *in vitro*. **(I)** Micropropagated melon plantlet. **(J)** Acclimated melon plant covered with a plastic pot to avoid dryness. **(K)** *In vivo* chromosome doubling treatment of a haploid plant by immersion of the apical meristem into a colchicine solution. **(L)** Melon fruit of a DH line. **(M)** Flow cytometry histogram of a haploid plant, showing ploidy-peaks at channels 150 and 300, corresponding to haploid cells in G0 phase and G2 phase, respectively. **(N)** Flow cytometry histogram of a mixoploid plant, showing ploidy-peaks at channels 150, 300, and 600, corresponding to haploid cells in G0 phase, diploid cells in G0 phase and haploid cells in G2 phase, and, diploid cells in G2 phase, respectively. **(O)** Flow cytometry histogram of a DH plant, showing ploidy-peaks at channels 300 and 600, corresponding to diploid cells in G0 phase and G2 phase, respectively.

DH Haploid Seed Recovery

The acclimated and chromosome doubled plants were grown in the greenhouse. When melon plants flowered, autopollination of DH plants was done by pollinating the emasculated female flowers with three to five male flowers. Once female flowers were pollinated, they were bagged and 3 days later the viability was checked. After 5 weeks since pollination melon fruits set were collected and DH seed was recovered.

Evaluation of Melon Fruit Traits

Melon fruit traits of donor genotypes were evaluated at commercial maturity stage. The evaluated traits were: fruit weight (FW); fruit diameter (FD); fruit length (FL); fruit shape (FL/FD); fruit skin netting (FSN), evaluated as 0 (no netting), 1 (low netting), 2 (moderate netting), 3 (high netting) and 4 (very high netting); sugar content (°Brix); and, fruit aroma, evaluated as 0 (no aroma), 1 (good aroma), 2 (very good aroma), and 3 (excellent aroma). The fruit aroma evaluation was done by a board of experts. Five to eight melon fruits were evaluated in each genotype.

Powdery Mildew, Fusarium Wilt, and MNSV Evaluation

The resistance or susceptibility of the seven donor genotypes was evaluated for *Podosphaera xhantii*, *Fusarium oxysporum* f.sp. *melonis*, and, MNSV. Five races of powdery mildew fungi *Podosphaera xhantii* (*Px*) were evaluated 1, 2, 3, 3.5, and 5. Fungi material was provided by GEVES (France). Plants were grown in the greenhouse until expansion of the third true leaf. Leaf disks of 9 cm diameters were taken from the first true leaf, disinfected in 20% sodium hypochlorite supplemented with 4 drops·L^{-1} of Tween 20 for 20 s, rinsed twice in sterile distilled water and plated into 90 mm petri dishes filled with powdery mildew medium, 25 mg L^{-1} of Benzimidazole and 1.6 g L^{-1} agar. Each leaf disk was inoculated manually with five conidiophores and up to ten leaf disks were analyzed in each genotype. After 12–15 days upon infection, each leaf disk was phenotypically evaluated according to the sporulation level using a scale from 0 to 4: 0, no sporulation; 1, hyphae present without no conidiophores and yellowing leaf disk; 2, hyphae present and up to twenty conidiophores; 3, 20–100 conidiophores present; 4, more than 100 conidiophores. Level 0 and 1 were considered as resistant, and levels 2–4 were considered as susceptible.

Resistance or susceptibility to *Fusarium oxysporum* f.sp. *melonis* (*Fom*) fungi races 0, 1, and 2 was evaluated. The fungi material was provided by BCCM (Belgium). Plants were grown in the greenhouse until third true leaf was expanded. Then, plants were removed from the substrate, the roots were washed with tap water and soaked for 30 s in a fungi solution of 3×10^6 spores·m L^{-1}. After the infection, plants were planted again in the substrate. Five plants were analyzed in each genotype. After 30 days upon infection, plants were evaluated according to fusarium wilt symptoms using a scale from 0 to 4: 0, plant without disease; 1, low levels of wilting and yellowing leaves; 2, leaves heavily affected by wilting; 3, all leaves wilted; 4, dead plant. Levels 0 and 1 were considered as resistant, and levels 2–4 were considered as susceptible.

For MNSV evaluation, plants were grown in the greenhouse until the expansion of the third true leaf. Then, virus inoculation was carried out by mechanical inoculation on the cotyledon with a solution of 0.03 M Na_2HPO_4, 0.2% DIECA, 75 g L^{-1} carborundum and 1 g of leaf infected with MNSV. Once the inoculation was made, plants were grown at 22°C and 50–70 μmol m^{-2} s^{-1} fluorescent light under a 16/8 h day/night photoperiod. Five plants were analyzed in each genotype. After 20 days upon infection, plants were evaluated according to the presence of virus symptoms using a scale from 0 to 3: 0, no symptoms present; 1, presence of few necrotic spots in the cotyledons; 2, presence of necrotic spots in cotyledons and true leaves, and malformation of new expanded leaves; 4, collapsed plant.

Statistical Analysis

The parameters FW, FD and FL were analyzed using ANOVA one-way test with a *post hoc* Bonferroni test ($P < 0.05$). The Brix (°) parameter was analyzed with Kruskal–Wallis with a *post hoc* Dunn's test ($P < 0.05$). The parameters FSN and fruit aroma and the parthenogenesis efficiencies were analyzed with Chi Square test ($P < 0.05$). All experiments were established in a completely randomized design.

RESULTS

Evaluation of Parental Donor Genotypes

Agronomic traits of the seven donor genotypes were evaluated in a random designed experiment during two consecutive years, 2017 and 2018 in test fields property of ROCALBA S.A. located in Monzón, Spain (**Table 1**). The fruit length (FL) of the evaluated genotypes ranged around 23 cm excepting Melito genotype which had a statistically different length ($P < 0.05$) of 19.82 ± 1.35 cm. FD showed significant differences between genotypes ($P < 0.05$), Melito had the smallest diameter and PS-0709 the widest. The shape parameter correlates FL and FD and therefore, express if fruits are ovate, elliptic, or elongated when higher or lower the ratio. The PS-0301 genotype showed the most elliptical melon fruits. The FW of melon fruits was relatively stable inside the same genotype. Nevertheless, significant differences ($P < 0.05$) between genotypes were found, Melito showed the lightest melon fruits (1.83 ± 0.35 kg) and PS-0809 the heaviest melon fruits (3.81 ± 0.77 kg). Sugar content, measured in Brix (°), and aroma are independent, higher values of sugar do not entail a better flavor. Melito and PS-1305 were considered as the best genotypes in terms of flavor because of the high values of aroma and sugar. No significant differences ($P > 0.05$) were found for the Kruskal–Wallis test analyzing FST and aroma due to a low number of replicates and the reduced range of the parameter. Pathogen resistance evaluation showed a high number of resistances in most of the genotypes excepting Melito, which was only resistant to *Fom* race 2 and sensitive to the other pathogens and races assayed. The genotypes PS-1305, PS-0301, PS-0709, PS-2001, and PS-2301 showed resistances for all *Px* and *Fom* races evaluated.

TABLE 1 | Fruit trait evaluation and pathogen resistance analysis of the seven genotypes of melon "Piel de Sapo."

	PS-1305	PS-1901	PS-0301	PS-0709	PS-2001	Melito	PS-2301
FL	23.53 ± 20^{a}	23.26 ± 1.86^{a}	24.88 ± 1.70^{a}	23.16 ± 1.94^{a}	22.81 ± 3.46^{a}	19.82 ± 1.35^{b}	23.82 ± 1.72^{a}
FD	14.28 ± 1.42^{abc}	15.63 ± 1.86^{abc}	15 ± 1.65^{abc}	17.37 ± 1.82^{d}	15.47 ± 2.13^{abc}	13.11 ± 1.35^{ac}	14.45 ± 1.71^{abc}
Shape	1.65	1.49	1.66	1.33	1.47	1.51	1.65
FW	2.58 ± 0.36^{a}	3.26 ± 0.69^{ab}	2.86 ± 0.50^{a}	3.81 ± 0.77^{bc}	3.29 ± 1.13^{ac}	1.83 ± 0.35^{d}	3.07 ± 0.59^{a}
FSN	1	2	2	2	1	0	1
Aroma	3	3	1	1	2	2	2
Brix (°)	14.17^{ab}	13.92^{abd}	12.31^{c}	13.47^{bd}	12.69^{cd}	14.47^{abd}	13.27^{bcd}
Fom 0	R	S	R	R	R	S	R
Fom 1	R	R	R	R	R	S	R
Fom 2	R	R	R	R	R	R	R
MSNV	R	S	S	R	S	S	R
Px 1	R	R	R	R	R	S	R
Px 2	R	R	R	R	R	S	R
Px 3	R	S	R	R	R	S	R
Px 3-5	R	R	R	R	R	S	R
Px 5	R	R	R	R	R	S	R

Values followed by a letter presented significantly differences ($P < 0.05$).

TABLE 2 | Parthenogenetic efficiencies of the seven genotypes of melon "Piel de Sapo."

Genotype	Pollinated flowers	Parthenogenesis induction with irradiated pollen				Germination and *in vitro* growth		Ploidy-level					
		Percentage of developed flowers*	Melon fruits	Embryos	Embryos/ melon fruit	Percentage of mortality	Embryos survived	*n*	(%)	*2n*	(%)	*n/2n*	(%)
PS-1305	165	10.30	17	6	0.35	33.33	4	3	75	1	25	0	0
PS-1901	143	15.38	22	3	0.14	33.33	2	1	50	1	50	0	0
PS-0301	130	24.62	32	12	0.38	75	3	2	66.67	1	33.33	0	0
PS-0709	196	9.69	19	5	0.26	40	3	3	100	0	0	0	0
PS-2001	169	18.34	31	6	0.19	33.33	4	2	50	2	50	0	0
Melito	202	17.82	36	16	0.44	56.25	7	5	71.43	1	14.29	1	14.29
PS-2301	123	17.07	21	5	0.24	40	3	3	100	0	0	0	0
Total	1,128	15.78*	178*	53	0.30*	50.94	26	19	73.08	6	23.08	1	3.84

*Parameters followed by * are significantly different ($P < 0.05$) between genotypes.*

However, resistance to MNSV was only present in PS-1305, PS-0709, and PS-2301.

Pollination With Irradiated Pollen and Parthenogenetic Embryo Rescue

Seven to eight plants of each genotype were grown in the greenhouse to be used as donor plant material (**Figure 1A**). A total of 1,128 flowers were pollinated with irradiated pollen and 178 of them developed melon fruit (**Figures 1C,D**). After pollination, some flowers initially developed but later failed to fruit set and finally aborted. A previous experiment had been carried out to analyze the germination of the irradiated pollen and the ability to fruit set. The irradiated pollen germinated correctly and set melon fruit when the female flower was pollinated the same and the next day upon irradiation. More days of storage or different storages reduced dramatically the germination of pollen and the fruit set (data not shown). Analyzing the seven genotypes, significant differences ($P < 0.05$) were found for melon fruit set between genotypes using a Chi Square test. The PS-0301 genotype had the highest fruit set (24.6%) and PS-0709 the lowest (9.7%) (**Table 2**). Melon fruits of 3 weeks old since pollination were collected and opened for parthenogenetic embryo rescue. Three different protocols were assayed to seek parthenogenetic embryos: one-by-one, X-ray radiography and floating seeds in liquid medium (**Table 3**). The seeds of 28 melon fruits were opened using the one-by-one method and eight parthenogenetic embryos were found. On the other hand, 127 melons were opened by X-ray radiography and 44 parthenogenetic embryos were found. Finally, 23 melons were opened by floating seeds in liquid medium and one parthenogenetic embryo was found. The percentage of detected melons carrying parthenogenetic embryos was similar between one-by-one and X-ray methods,

TABLE 3 | Parthenogenetic embryo rescue methods assayed for embryo detection and rescue.

Method detection	Melons opened	Embryos rescued*	Ratio of fruits containing embryo
One-by-one	28	8	0.28
X-Ray	127	44	0.34
Liquid medium	23	1	0.04

*Parameters followed by * are significantly different ($P < 0.05$) between methods.*

28.57 and 34.65%, respectively. Nevertheless, X-ray method was found to be four to five-times faster than one-by-one method due to seeds containing parthenogenetic embryo were the only ones opened (**Figures 1E,F**). When floating seeds in liquid medium, only 4.35% of melon fruits were contained parthenogenetic embryos due to many of the cultured seeds in liquid medium were contaminated and therefore discarded, despite the initial decontamination of seeds with bleach. Overall, a total of 53 parthenogenetic embryos (**Figure 1G**) were rescued from the 178 melon fruits set (**Table 2**). Parthenogenetic embryos were rescued in all donor genotypes, the highest number of parthenogenetic embryos found per genotype was 16, in Melito and the lowest 3, in PS-1901. The ratio of parthenogenetic embryos rescued per melon fruit ranged between 0.14 and 0.38. Although all melon fruits carried a normal number of seeds (between 300 and 500) the 71.91% of fruits had no parthenogenetic embryos. The 53 haploid embryos were recovered from 50 melon fruits. No significant differences ($P > 0.05$) were found using a Chi Square test for the number of parthenogenetic embryos among genotypes.

The parthenogenetic embryos rescued were transferred to solid E20A medium for germination and further plantlet development (**Figures 1H,I**). From 53 embryos, six failed to germinate, appearing a necrosis in the cotyledonary embryos at the second or third week since rescue. From the 47 germinated embryos, 21 plantlets suffered a stagnation of development and died (**Table 2**). No significant differences were found between genotypes for embryo germination and plantlet development ($P > 0.05$). Thus, 26 parthenogenetic independent-genotypes plantlets were able to grow *in vitro* and micropropagation was carried out until greenhouse acclimation (**Figure 1J**).

Ploidy-Level and Chromosome Doubling

In order to maximize the number of DH plantlets and to ensure the recovery of seeds from DH genotypes, ploidy-level of the parthenogenetic germinated plantlets was analyzed prior to chromosome doubling. Parthenogenesis was found to be successful since haploid, spontaneous DH and mixoploid plantlets were recovered (**Table 2**).The ploidy-level of the 26 parthenogenetic lines was analyzed by flow cytometry and showed that the 73% were haploid (**Figure 1M**), the 23% were spontaneous DH (**Figure 1N**), and one plantlet was found to be mixoploid (**Figure 1O**). The six spontaneous DH plants were acclimatized in the

TABLE 4 | Chromosome doubling protocols assayed.

Treatment			Explants or apical meristems treated	Mortality (%)*	Ploidy-level				Acclimated plants	Melon fruits recovered
Antimitotic compound	Concentration (mg L^{-1})	Time (h)			*n*	%	*n/2n*	%	*2n*	%		
Colchicine *in vitro*	500	12	67	86.57	7	36.84	12	63.16	0	0	2	0
Oryzalin *in vitro*	50	18	47	95.74	1	50	0	0	1	50	1	0
Colchicine *in vivo*	5,000	2	150	42.67	45	70.31	6	9.38	13	20.31	–	12

*Parameters followed by * are significantly different ($P < 0.05$) between methods.*

greenhouse, if they produced pollen and no chromosome doubling was applied. The mixoploid line presented a high ratio of haploid male flowers without pollen and was treated as a haploid.

Chromosome doubling of the 20 haploid parthenogenetic plants was done using different protocols to establish the most efficient one (**Table 4**). Two antimitotic compounds, colchicine and oryzalin, were assayed for *in vitro* chromosome doubling. On the other hand, colchicine was used for *in vivo* chromosome doubling. A total of 114 nodules or shoot tips were treated *in vitro*, 67 and 47, for colchicine and oryzalin, respectively. *In vitro* colchicine treatment resulted in a high number of dead nodules and shoot tips, 86.57%. From the survived plantlets, only two were successfully chromosome doubled and survived the acclimatazion. *In vitro* oryzalin treatment had a lower short-term death, 89.36% of the nodules or shoot tips survived the next 2 weeks since the chromosome doubling treatment and developed two to three new leaves. Nevertheless, from the 42 survived nodules and shoot tips, 41 presented a high level of hyperhidricity in the base of the nodules or shoot tips that impeded root growing and therefore, no acclimatazion was possible. Final rate of mortality was 95.74%. Only one plantlet was successfully chromosome doubled and acclimatized.

Due to the low values of the chromosome doubling *in vitro* treatments, *in vivo* chromosome doubling was assayed. Haploid plantlets were acclimatized in the greenhouse (**Figure 1K**). *In vivo* chromosome doubling was done using colchicine as the antimitotic agent. A total of 150 plant tips were treated with colchicine and 57.33% survived the treatment. From survived plants, 69.77% remained haploid, 9.30% were mixoploids and 20.93% successfully chromosome doubled. The ploidy of chromosome doubled plants, which was analyzed by flow cytometry, was re-checked phenotypically to uphold the successful duplication of plants by checking the presence of pollen in male flowers.

DH Seed Recovery and Pollen Counts

To recover DH seed, spontaneous DH lines and chromosome doubled lines were autopollinated (**Figure 1L**). The 33% of DH lines presented male flowers with pollen together with haploid male flowers without pollen, those plants were classified as phenotypically mixoploid although being detected as pure DH by flow cytometry. No fruit recovery was possible from the *in vitro* chromosome doubled plants. From the *in vivo* duplicated plants, a total of twelve melon fruits were recovered from eight independent parthenogenetic DH lines (**Table 5**). A total of 372 female flowers were pollinated and the fruit set was 3.23%. Genotypes from which no melon fruit was recovered fruit set was impossible. Three out of twelve melon fruits, DH2-PS-2001 and DH8-Melito, carried empty seeds. One chromosome doubled plant, DH4-Melito, did not develop male neither female flowers and no pollination was possible. Finally, DH seed was obtained from six DH plants: DH11-PS-1305, DH3-PS1901, DH9-DH0301, DH5-PS0709, DH10-Melito, and DH1-PS-2301.

DISCUSSION

Doubled haploid technology has entailed a great progress in plant breeding because of the production of homozygous lines in a shorter time compared to traditional breeding. In *Cucurbitaceae*, DHs are usually produced for commercial means, either to be used as homozygous stable cultivars or as parental pure lines for hybrid F1 seed production. Thanks to heterosis, hybrid F1 cultivars have enhanced traits than their own parental lines. In this work, the donor material was a batch of seven genotypes of melon "Piel de Sapo" type evaluated and characterized for their agronomic traits and pathogen resistances. Later on, their parthenogenetic potential was evaluated focusing on pollination with irradiated pollen, parthenogenetic embryo rescue, *in vitro* plantlet performance and chromosome doubling. The production and the consumption of melon "Piel de Sapo" type is localized mainly in Spain due to cultural reasons where it has a high commercial value because of its differentiated quality. Besides, Spain is the eighth country in terms of melon fruit production worldwide and is the first country in terms of exportation to Europe. We attempted to obtain DH lines of melon with the aim to use them as parental donor lines for commercial hybrid F1 cultivars.

The agronomic traits of melon fruits and the pathogen resistances of the donor material were evaluated in order to analyze the potential use of parthenogenetic-derived DH lines as parental for melon "Piel de Sapo" hybrid F1 cultivars. The agronomic results showed a great variability of melon fruit parameters between the seven evaluated genotypes. Although Melito inbred cultivar presented low pathogen resistances its melon fruits were valuable because of its small dimensions and the high aroma and sucrose content. Monoecious plants are more likely to have elongated fruits (Robinson, 2000), and PS-0301 genotype was monoecious and presented more elongated fruits in comparison to the other six genotypes. The °Brix and aroma of melon fruits were not always correlated. Flavor depends upon taste (sweetness and acidity) and aroma. Besides, °Brix only measures the concentration of predominant sugars, as fructose, sucrose and glucose, and organic acids. Aroma is often considered to play a dominant role in flavor of fruits and vegetables and is dependent upon low-molecular-weight-volatile compounds as largely esters, alcohols, aldehydes and ketones, which are not measured with the refractometer (Kader, 2008). Therefore, melon fruits of PS-1305, PS-1901, and Melito were considered as the best ones in terms of balance between aroma and sugar content. The majority of genotypes assayed presented pathogen resistances. Pathogen resistance or susceptibility to *Podosphaera xhantii*, causing powdery mildew, *Fusarium oxysporum* f.sp. *melonis*, causing fusarium wilt, and MNSV was evaluated because are the major diseases in melon. The use of resistant cultivars is the best approach to control pathogen spreading and disease. In the southern of Europe, *Podosphaera xhantii* races 1, 2, and 5 are the most frequent (Yuste-Lisbona et al., 2010). Although powdery mildew can be controlled by fungicides its long-term use led to fungicide resistance of powdery mildew. The use of resistant cultivars is a more effective and environmentally safe way to control the

TABLE 5 | Melon fruits recovered from DH lines.

Doubled haploid line	Number of clones	Pollinated flowers	Phenotypical ploidy	Pollen observations	Melon fruits
DH11-PS-1305	5	58	Diploid	Normal male flowers	1
DH3-PS1901	3	47	Diploid	Normal male flowers	2
DH9-DH0301	4	62	Diploid	Small size male flowers with less pollen	1
DH5-PS0709	2	28	Diploid	Normal male flowers	1
DH2-PS-2001	2	36	Diploid	Indehiscent pollen	2
DH8-Melito	3	42	Diploid	Normal male flowers	1
DH10-Melito	3	54	Mixoploid	Normal and haploid male flowers	1
DH1-PS-2301	4	45	Diploid	Normal male flowers	3

disease. On the other hand, *Fusarium oxysporum* f.sp. *melonis* is one of the most difficult diseases to control because the pathogen is soil-borne and remains viable in the soil as chlamydospores (Joobeur et al., 2004). Concerning to MNSV, the best source of resistance in melon is the *nsv* gene, which confers a recessive resistance to MNSV (Nieto et al., 2007). Therefore, genotypes such as PS-1305, PS-0709, and PS-2301, were considered as the best genotypes it terms of pathogen resistance.

The genotype of the donor material has a crucial influence for the success of DH protocols as reported in many species, including melon. Parthenogenesis in *inodorus* "Piel de Sapo" type genotypes has been reported once and had not been much studied in comparison to other genotypes such as *inodorus* "Galia" type, *chinensis* or *cantalupensis*, possibly because of its local importance. In this parthenogenetic study, the genotypic response of seven genotypes of melon "Piel de Sapo" type differed for: fruit set when pollinated with irradiated pollen; parthenogenetic embryo induction; haploid embryo germination; chromosome doubling; and, fruit set of DH lines. The parthenogenetic ability of melon "Piel de Sapo" germplasm used was lower than other genotypes such as *chinensis*, *cantalupensis* or *inodorus* (Lotfi et al., 2003; Lim and Earle, 2008; Gonzalo et al., 2011).

Fruit set of donor plants after pollination with irradiated pollen is the first step of *in situ* parthenogenesis. In *Cucurbitaceae*, *in situ* parthenogenesis induction through gamma-ray irradiated-pollen has been achieved in melon, cucumber, watermelon and winter squash since the first report of Sauton and Dumas de Vaulx (1987). Nevertheless, no reports focus on the efficiency of the pollination with irradiated pollen. In this study, the number of pollinated flowers with irradiated pollen and its later development or drop was recorded. The efficiency of pollination varied between genotypes, the lowest value was 9.69% and the highest 24.62%, in PS-0709 and PS-0301, respectively. The low number of developed female flowers (15.78% in average) and fruit set is attributable to the irradiation process suffered by the pollen. Although irradiated pollen can germinate on the stigma and grow within the style reaching the embryo sac is genetically inactivated to fertilize the egg-cell and the polar nuclei. Therefore, irradiated pollen stimulates egg-cell division and induces haploid embryos (Cuny, 1992). Pollen sensitivity to irradiation its attributed to radio-resistance, and the viability of pollen is decreased along with the irradiation exposure. Previous reports in melon (Lim and Earle, 2008; Gonzalo et al., 2011; Godbole and Murthy, 2012) used an irradiation exposure of 250 Gy, therefore, prior to the experiment, this dose was evaluated based on fruit set and pollen germination assays (data not shown). Moreover, during pollination with irradiated pollen, fruit set was observed to be dependent on: the time of the year, being August the period when more fruit set; the stage of donor plants, at the beginning of flowering and the end of the greenhouse culture fruit set was low; and, the weather, cloudy pollination days resulted in less fruits than shinny days. Pollen storage viability was evaluated through the pollination with irradiated male flowers with zero, one and two days since irradiation. The storage of irradiated male flowers in plastic pots in darkness for one day was successful to maintain pollen viability. Therefore, pollen could be used to pollinate female flowers. More than one day of storage resulted in a decrease of pollen viability and inability to set melon fruits.

The parthenogenetic embryo production was reported to be genotypic dependent. From all genotypes, a total of 178 melon fruits and 53 embryos were obtained. Normally, the parthenogenetic embryo efficiency is expressed as embryos per seed. Nevertheless, the process of detecting embryos is tedious enough to additionally count the seeds. In this study, the efficiency was expressed as parthenogenetic embryos contained per fruit. The ratio of embryos per fruit in melon "Piel de Sapo" ranged between 0.14 and 0.44, similar to the 0–3 reported in genotypes of "Piel de Sapo" by Gonzalo et al. (2011). Besides, it was lower than the ratio of 4–18 in *inodorus* genotype of the "Galia" type reported by Lotfi et al. (2003) and the high ratio of 16 reported by Lim and Earle (2008). About one third of melon fruits contained at least one embryo, meaning that the vast majority of melon fruits had an average of 400 empty seeds. The parthenogenetic embryo detection process is laborious and time-consuming, the results are very inefficient compared with the time invested. Because of this, different methodologies have been described to detect parthenogenetic embryos, being the inspection of seeds one-by-one the most commonly applied (Chun et al., 2006; Smiech et al., 2008; Godbole and Murthy, 2012), followed by X-ray radiography of seeds (Dolcet-Sanjuan et al., 2004; Claveria et al., 2005) and the culture of seeds in liquid medium (Lotfi et al., 2003). In this study, three methods were assayed in order to reduce the amount of time and work invested during the process of embryo detection without compromising the embryo itself. Although seed culture in liquid medium reduced drastically the amount of work it was not effective and compromised the parthenogenetic embryo because

of endophytic bacterial and fungi contaminations, despite the initial sterilization of seeds. On the other hand, one-by-one and X-ray methods resulted in a similar ratio of embryo per melon, 0.28 and 0.35, showing that both did not compromise the obtention of embryos. Nevertheless, X-ray method was five time faster than one-by-one method. Then, X-ray method was selected for routine laboratory use.

Once parthenogenetic embryos were detected, they were cultured *in vitro* for germination, development and micropagation. From 53 rescued embryos, 26 germinated, grew *in vitro* and developed plantlets. The 11.32% of embryos failed to germinate and from those germinated, the 39.62% died before the first micropropagation was possible because failed to grow and did not develop the first true leaf. *In vitro* germination and growth are critical steps that can jeopardize the *in situ* parthenogenetic process. Deleterious gene combination in homozygosity regulating vegetative growth may be responsible of hampering germination and plantlet development (Geoffriau et al., 1997). During the *in vitro* process there is a high selection pressure impeding the survival of embryos with deleterious recessive alleles in homozygosity (Cuny, 1992). The results showed a mortality of 25–66.67% depending on the genotype and is in accordance with other authors, reporting a 42–62% (Lim and Earle, 2008) or 62–84% (Lotfi et al., 2003).

To restore diploid chromosome content in haploid melon plants, induced chromosome doubling is mandatory. In cucurbits, the number of spontaneous doubled haploids obtained during DH methodology is usually low compared with other species, that can represent the 30–55% of androgenetic plants in bell pepper (Irikova et al., 2011; Keleş et al., 2015) or 30% in rice (Hooghvorst et al., 2018). The ploidy-level of the produced plants was analyzed: 73% were haploid, 4% mixoploid and 23% spontaneous chromosome doubled. The ploidy-level results presented are in accordance with those of Lim and Earle (2008), who found a 73% of haploids and 27% mixoploids in melon; or Kurtar and Balkaya (2010), that produced 76.71% spontaneous DHs and 23.29% haploids in squash; or, Sauton (1988, 1989), who reported spontaneous doubling in cucumber and melon for the first time. Spontaneous duplication can occur when endomitosis or nuclear fusion happens. In endomitosis process, cell multiplicate chromosomes and separate them in each cell pole during early mitosis, nevertheless, cell fails to divide, and two sets of chromosomes is restituted. In nuclear fusion, two or more synchronized nuclei divide and develop a common spindle (Kasha, 2005). Spontaneous DHs and mixoploids have an endomitotic or nuclei fusion origin. Notwithstanding, in spontaneous DHs, the endomitosis or nuclear fusion took place at early stages of the development of the egg-cell, and in mixoploid plants occurred later, causing a different ploidy-level of the germ cells. Then, induced chromosome doubling of haploid plants is necessary prior to DH seed recovery. Colchicine is the most used antimitotic for chromosome doubling in DH technology. When haploids are treated with antimitotic compounds a so-called C-mitosis can take place. During interphase, cells have their chromosomes duplicated with the chromatid sisters placed in each pole of the cell bound by the centromere' spindle tubule. The antimitotic compound interacts with the tubulin subunits and destabilize the spindle tubule arresting cells during interphase. Chromosome doubling is a required step in parthenogenesis DH protocols in *Cucurbitaceae*. Three different protocols were assayed for chromosome doubling, two protocols *in vitro* with colchicine or oryzalin, and one *in vivo* with colchicine. *In vitro* protocols resulted in a high mortality rate due to antimitotic toxicity. The recorded chromosome duplication efficiency of 500 mg L^{-1} of colchicine for 12 h *in vitro* treatment was 63.16%. However, majority of plantlets failed to develop after the treatment and before the ploidy-level analysis. The rate of chromosome doubled and successfully acclimatized plants was 2.98% (two plants). Similar results were recorded for 50 mg L^{-1} of oryzalin for 18 h *in vitro* treatment which resulted in one plant (2.13%) survived and chromosome doubled. Those results show a high sensitivity of the genotypes to the antimitotic compounds and to the *in vitro* culture once treated, which do not line with other reported studies that show *in vitro* chromosome doubling treatment as successful and the preferred (Dong et al., 2016; Ebrahimzadeh et al., 2018). This could be explained as a recalcitrant performance of the "Piel de Sapo" genotypes not only to the antimitotic treatment but also to *in vitro* culture, which in turn resulted in a low production of DH lines. Due to the low efficiency of survival and chromosome doubling of *in vitro* treatments, *in vivo* 5,000 mg L^{-1} of colchicine for 2 h on apical meristems was assayed. Resulting in 69.77% of survival and 13.33% of chromosome doubling, being 8% DHs and 5.33% mixoploids. Other authors have reported higher chromosome duplication efficiencies when treating *in vivo* with colchicine as 46.03% in "Kirkagac" and "Yuva Hasanbey" melon genotypes (Solmaz et al., 2011) or 19% (Lim and Earle, 2008). The *in vivo* chromosome doubling efficiencies were acceptable in spite of being low, those are in line with the low *in vitro* efficiencies and other parameters analyzed previously, which support the hypothesis of the recalcitrant performance of "Piel de Sapo" genotypes during the entire parthenogenetic process. The fruit set of the 20 DH lines and the eight mixoploid lines was low. A total of 12 fruits from eight independent parthenogenetic DHs were recovered. Pollination of induced chromosome doubled plants was dramatically difficult, an average of 3.23% of pollinated female flowers set fruit. This ratio was even lower than the pollination with irradiated pollen. From the fruits recovered, both of DH2-PS-2001 and the one of DH10, contained the usual amount of seeds despite all of them were empty. Consequently, DH seed was not recovered from those genotypes. Lim and Earle (2008) proved that pollen viability of chromosome doubled plants its usually low, affecting fruit set and seed viability. In their study, they recommend *in vitro* chromosome doubling because had higher pollen germination rates than *in vivo* chromosome doubled plants. We had no success in *in vitro* chromosome doubling, and the *in vivo* chromosome doubled plants had a low capacity to set fruits.

In this study, "Piel de Sapo" donor material had traits with potential value for commercial purposes such as melon fruit morphology, sweetness and aroma, and pathogen resistances against important diseases such as powdery mildew, fusarium wilt and MNSV virus. Moreover, the *in situ* parthenogenetic

of endophytic bacterial and fungi contaminations, despite the initial sterilization of seeds. On the other hand, one-by-one and X-ray methods resulted in a similar ratio of embryo per melon, 0.28 and 0.35, showing that both did not compromise the obtention of embryos. Nevertheless, X-ray method was five time faster than one-by-one method. Then, X-ray method was selected for routine laboratory use.

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To restore diploid chromosome content in haploid melon plants, induced chromosome doubling is mandatory. In cucurbits, the number of spontaneous doubled haploids obtained during DH methodology is usually low compared with other species, that can represent the 30–55% of androgenetic plants in bell pepper (Irikova et al., 2011; Keleş et al., 2015) or 30% in rice (Hooghvorst et al., 2018). The ploidy-level of the produced plants was analyzed: 73% were haploid, 4% mixoploid and 23% spontaneous chromosome doubled. The ploidy-level results presented are in accordance with those of Lim and Earle (2008), who found a 73% of haploids and 27% mixoploids in melon; or Kurtar and Balkaya (2010), that produced 76.71% spontaneous DHs and 23.29% haploids in squash; or, Sauton (1988, 1989), who reported spontaneous doubling in cucumber and melon for the first time. Spontaneous duplication can occur when endomitosis or nuclear fusion happens. In endomitosis process, cell multiplicate chromosomes and separate them in each cell pole during early mitosis, nevertheless, cell fails to divide, and two sets of chromosomes is restituted. In nuclear fusion, two or more synchronized nuclei divide and develop a common spindle (Kasha, 2005). Spontaneous DHs and mixoploids have an endomitotic or nuclei fusion origin. Notwithstanding, in spontaneous DHs, the endomitosis or nuclear fusion took place at early stages of the development of the egg-cell, and in mixoploid plants occurred later, causing a different ploidy-level of the germ cells. Then, induced chromosome doubling of haploid plants is necessary prior to DH seed recovery. Colchicine is the most used antimitotic for chromosome doubling in DH technology. When haploids are treated with antimitotic compounds a so-called C-mitosis can take place. During interphase, cells have their chromosomes duplicated with the chromatid sisters placed in each pole of the cell bound by the centromere' spindle tubule. The antimitotic compound interacts with the tubulin subunits and destabilize the spindle tubule arresting cells during interphase. Chromosome doubling is a required step in parthenogenesis DH protocols in *Cucurbitaceae*. Three different protocols were assayed for chromosome doubling, two protocols *in vitro* with colchicine or oryzalin, and one *in vivo* with colchicine. *In vitro* protocols resulted in a high mortality rate due to antimitotic toxicity. The recorded chromosome duplication efficiency of 500 mg L^{-1} of colchicine for 12 h *in vitro* treatment was 63.16%. However, majority of plantlets failed to develop after the treatment and before the ploidy-level analysis. The rate of chromosome doubled and successfully acclimatized plants was 2.98% (two plants). Similar results were recorded for 50 mg L^{-1} of oryzalin for 18 h *in vitro* treatment which resulted in one plant (2.13%) survived and chromosome doubled. Those results show a high sensitivity of the genotypes to the antimitotic compounds and to the *in vitro* culture once treated, which do not line with other reported studies that show *in vitro* chromosome doubling treatment as successful and the preferred (Dong et al., 2016; Ebrahimzadeh et al., 2018). This could be explained as a recalcitrant performance of the "Piel de Sapo" genotypes not only to the antimitotic treatment but also to *in vitro* culture, which in turn resulted in a low production of DH lines. Due to the low efficiency of survival and chromosome doubling of *in vitro* treatments, *in vivo* 5,000 mg L^{-1} of colchicine for 2 h on apical meristems was assayed. Resulting in 69.77% of survival and 13.33% of chromosome doubling, being 8% DHs and 5.33% mixoploids. Other authors have reported higher chromosome duplication efficiencies when treating *in vivo* with colchicine as 46.03% in "Kirkagac" and "Yuva Hasanbey" melon genotypes (Solmaz et al., 2011) or 19% (Lim and Earle, 2008). The *in vivo* chromosome doubling efficiencies were acceptable in spite of being low, those are in line with the low *in vitro* efficiencies and other parameters analyzed previously, which support the hypothesis of the recalcitrant performance of "Piel de Sapo" genotypes during the entire parthenogenetic process. The fruit set of the 20 DH lines and the eight mixoploid lines was low. A total of 12 fruits from eight independent parthenogenetic DHs were recovered. Pollination of induced chromosome doubled plants was dramatically difficult, an average of 3.23% of pollinated female flowers set fruit. This ratio was even lower than the pollination with irradiated pollen. From the fruits recovered, both of DH2-PS-2001 and the one of DH10, contained the usual amount of seeds despite all of them were empty. Consequently, DH seed was not recovered from those genotypes. Lim and Earle (2008) proved that pollen viability of chromosome doubled plants its usually low, affecting fruit set and seed viability. In their study, they recommend *in vitro* chromosome doubling because had higher pollen germination rates than *in vivo* chromosome doubled plants. We had no success in *in vitro* chromosome doubling, and the *in vivo* chromosome doubled plants had a low capacity to set fruits.

In this study, "Piel de Sapo" donor material had traits with potential value for commercial purposes such as melon fruit morphology, sweetness and aroma, and pathogen resistances against important diseases such as powdery mildew, fusarium wilt and MNSV virus. Moreover, the *in situ* parthenogenetic

capacity of "Piel de Sapo" germplasm was evaluated, showing: a low capacity of fruit set when pollinated with irradiated pollen, a low production of parthenogenetic embryos, a poor *in vitro* culture performance, a low chromosome doubling and a low fruit set of DH lines once chromosomes were doubled. The "Piel de Sapo" *inodorus* type can be considered as a recalcitrant genotype for parthenogenesis in melon species in comparison to other genotypes. Nevertheless, we succeed to obtain DH seed that have a great value for hybrid F1 seed production and commercialization. During the parthenogenetic process, X-ray method was concluded as the most successful and optimum method to detect and rescue parthenogenetic embryos. The poor performance of "Piel de Sapo" genotypes showed during *in vitro* culture could be enhanced changing media composition instead of using the traditional E20A medium. In addition, an *in vivo* chromosome doubling method with colchicine was adapted and resulted as the most successful for chromosome doubling of haploid plants, in front of *in vitro* chromosome doubling methods with colchicine or oryzalin. Although parthenogenetic DH plants were obtained from six out of seven melon "Piel de Sapo" genotypes further improvements of the process using variations should be assayed in order to produce a higher number of DH plants that could be used in melon breeding programs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

IH designed, supervised, and participated in all the experiments and wrote the manuscript. OT participated during the first stage of parthenogenesis process and helped writing the manuscript. SH participated and supervised the agronomic and pathogen evaluation experiments and corrected the manuscript. SN helped during all parthenogenetic process and manuscript correction.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 2. Efficient knockout of phytoene desaturase gene using CRISPR/Cas9 in melón

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Efficient knockout of phytoene desaturase gene using CRISPR/Cas9 in melon

Isidre Hooghvorst[1,2*], Camilo López-Cristoffanini[1] & Salvador Nogués[1]

CRISPR/Cas9 system has been widely applied in many plant species to induce mutations in the genome for studying gene function and improving crops. However, to our knowledge, there is no report of CRISPR/Cas9-mediated genome editing in melon (*Cucumis melo*). In our study, phytoene desaturase gene of melon (*CmPDS*) was selected as target for the CRISPR/Cas9 system with two designed gRNAs, targeting exons 1 and 2. A construct (pHSE-CmPDS) carrying both gRNAs and the Cas9 protein was delivered by PEG-mediated transformation in protoplasts. Mutations were detected in protoplasts for both gRNAs. Subsequently, *Agrobacterium*-mediated transformation of cotyledonary explants was carried out, and fully albino and chimeric albino plants were successfully regenerated. A regeneration efficiency of 71% of transformed plants was achieved from cotyledonary explants, a 39% of genetic transformed plants were successful gene edited, and finally, a 42–45% of mutation rate was detected by Sanger analysis. In melon protoplasts and plants most mutations were substitutions (91%), followed by insertions (7%) and deletions (2%). We set up a CRISPR/Cas9-mediated genome editing protocol which is efficient and feasible in melon, generating multi-allelic mutations in both genomic target sites of the *CmPDS* gene showing an albino phenotype easily detectable after only few weeks after *Agrobacterium*-mediated transformation.

Genome editing tools have the potential to modify genomic sequences with accuracy. Some of these tools are: homologous recombination (HR), targeted induced local lesions in the genome (TILLING), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), or clustered regularly interspaced short palindromic repeats associated to nuclease Cas9 (CRISPR/Cas9). ZFN, TALENs and CRISPR/Cas9 are site-specific nucleases. The CRISPR/Cas9 genome editing tool was developed in 2013, and in comparison with other genome editing tools has better efficacy, efficiency, versatility and is simpler[1].

CRISPR/Cas9 system cleaves a specific region of DNA by the Cas9 nuclease, which is guided by a 20-nt sequence named RNA-guide (gRNA). The association between Cas9 and gRNA, and the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA (typically NGG), allows a precise editing of DNA target sequences. The endonuclease domain induces DNA double-strand breaks (DSB), which can be repaired by either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) generating insertions and deletions events (INDELs) and substitutions[2,3]. The major uses of CRISPR/Cas9 in plants have been gene knockouts to elucidate the function of a target gene by gene mutation and transcriptional regulation[4]. This application allows genes function studies, to knock out genes that negatively affect food quality, to confer resistance to pathogens or divert metabolic flux away from valuable end-products[1].

CRISPR/Cas9 system is theoretically applicable to all plant species, but many of them lack the experimental demonstration of its applicability. Since 2013, CRISPR/Cas9 has been applied in *Oryza sativa*[5], *Arabidopsis thaliana*[6], *Nicotiana benthamiana*[7], *Solanum lycopersicum*[8], *Zea mays*[9] and soybean[10], among other species. The strategy to mutate and knockout the phytoene desaturase gene (*PDS*) by CRISPR/Cas9 has been widely applied to quickly demonstrate the feasibility of CRISPR/Cas9 since its mutation causes photobleaching or albino phenotype. Through transient expression assays or transformation methods, CRISPR/Cas9 mutations have been demonstrated and studied in plants. Within the *Cucurbitaceae* family, this genome editing technique has only been reported as successfully applied in cucumber[11] and watermelon[12,13].

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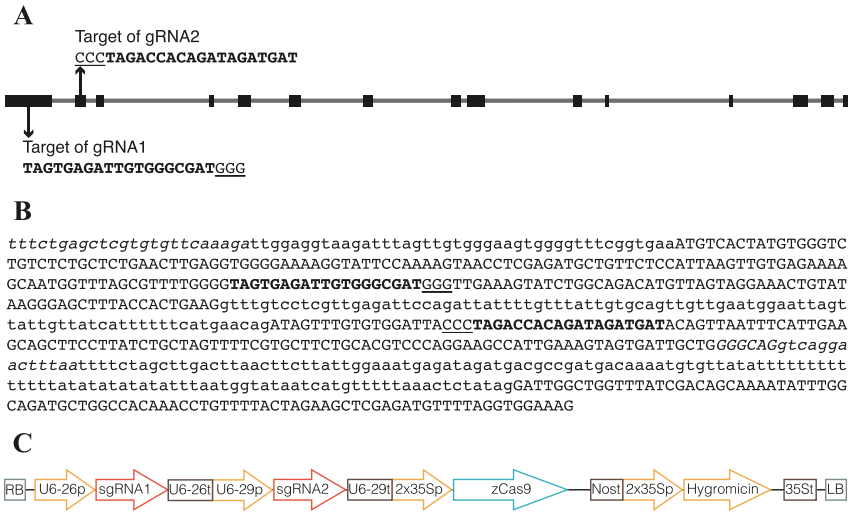

Figure 1. Schematic representations of the melon *CmPDS* target gene (MELO3C017772.2 in ICUGI data base), location of the gRNA1 and gRNA2, and the CRISPR/Cas9 vector. (**A**) Schematic representation of melon *CmPDS* gene with two target sites indicated as bold and PAM sequences as underlined. (**B**) Representation of *CmPDS* target sequences. Exons are shown as capital letters, introns as lowercase, gRNA1 and gRNA2 as bold, PAM sequences as underlined, and primers used for gene sequencing as italics. (**C**) Schematic representation of the CRISPR/Cas9 binary vector used for melon transformation. *Arabidopsis thaliana* promoters and terminators drive expression of gRNA1(AtU6–26p and AtU6–26t) and gRNA2 (AtU6–29p and AtU6–29t). The Cauliflower mosaic virus promoter (CaMV 35S) drives the expression of the Cas9 gene.

Melon (*Cucumis melo*) belongs to *Cucurbitaceae* family and is an important plant because of its specific biological properties and economic value of its fruit. In fact, 31 million tons of melon were produced worldwide in 2016[14], being an important crop in Mediterranean and East Asian countries. The recent melon genome publication by Garcia-Mas *et al.*[15] and the versatility of CRISPR/Cas9 allows to study and explore gene functions in melon. However, to our knowledge, CRISPR/Cas9 application in melon has not been reported. Therefore, the aim of this study was to demonstrate, for the first time, the applicability of CRISPR/Cas9 system on melon by performing a gene knockout of the melon phytoene desaturase gene (*CmPDS*) in protoplasts and plants.

Results

Target selection and vector construction for CRISPR/Cas9 system. To test the efficacy of CRISPR/Cas9 system in melon, we chose to disrupt phytoene desaturase gene of *Cucumis melo* (CmPDS) which has a single copy, located in the chromosome 7, with reference MELO3C017772.2 in ICUGI data base. The genomic sequence of this reference is 10,443 bp in size, with 14 exons (Fig. 1A). Target sites in *CmPDS* were designed using Benchling[16] and two target sites were selected: gRNA1 and gRNA2, in exon 1 and 2 respectively (Fig. 1A). Both gRNAs were cloned into one binary vector (pHSE-CmelPDS) carrying the promoter for the Cas9 gene (CaMV 35S), the Cas9 gene, AtU6–26p and AtU6–29p promoters and gRNA scaffold (Fig. 1C). Potential off-target sites were searched using CRISPR-OFFinder[17], and no potential off-target were detected with 0 to 2 mismatches (Table S1)[18].

Targeted mutagenesis in melon protoplasts. CRISPR/Cas9 vector pHSE-CmelPDS was tested in protoplasts to validate the functionality of Cas9 via transient expression by PEG-mediated protoplast transfection. A total of 24 protoplast cell colonies were Sanger-sequenced. According to the number of mutated colonies detected, the target efficiency for pHSE-CmelPDS in melon protoplasts was 25% for both gRNAs. Moreover, most of the mutations analyzed were substitutions and two nucleotides insertions (Fig. 3 and Table S2). These results suggested that the vector was viable to be used in melon for plant regeneration.

PDS-edited plants phenotype. Alteration of *CmPDS* gene expression function was manifested as albino and dwarf plants (Fig. 2). Some cotyledons only regenerated albino shoots, meanwhile others regenerated green shoots and after a while exhibited mosaicism or regenerated secondary albino shoots. Complete albino plants exhibited a high level of dwarfism, having a reduced leaf area, a growth lesser than 0.5 cm, and impossibility to perform *in vitro* propagation. A total of 77 plants were regenerated from 958 *Agrobacterium*-mediated transformed cotyledonary explants. The 22.07% of the regenerated plants showed albino phenotype, 4 complete albino plants and 13 chimeric albinism were detected. Albino plants did not survive more than 3 months after regeneration.

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WT gRNA1 GTGAGAAAAGCAATGGT·TTAGCGTTTTGGGG·TAGTGAGATTGTGGGCGATGGGTTGAAAGTATCTGGCAGACATGTTAGTA
P  (1)   GTGAGAAAAGCAATGGT·TTAGCGTTTTGGGG·TAGTGAGATTCTGGGCGATGGGTTGAAAGTATCTGGCAGACATGTTAGTA  S1
P  (1)   GTGAGAATAGCAATGGT·TTAGCGTTTTGGGG·TAATGAGATTGTGGGCGATGGGTTGAAAGTACCTGGCAGACATGTTAGTA  S3
42 (3)   GTGAGAAAAGCAATGGT·TTAGCGTTTTGGGG·TAGTGTGATTGTGGGCGATGGGTTGAAAGTATCTGGCAGACATGTTAGTA  S1
44 (2)   GTGAGAAAAGCAATGGT·TTAGCG-TTTGGGG·TAGTGAGATTGTGGGCGATGGGTTGAAAGTATCAGGCAGACATGTTAGTA  -1,S1
44 (4)   ATGAGAAAAGCAATGGT·TTAGCGTTTTGGGG·TAGTGAGATTGTGGGCGGTGGGTTGAAAGTATCTGGCAGACATGTTAGTA  S2
46 (5)   GTGAGAAAAGCAATGGT·TTAGCGTTTTGGGG·TAGTGAGATTGTGGGCGATGGGTTGAAAGTATCTGGCAGACACGTTAGTA  S1
50 (2)   GTGAGAAAAGCAATGGTATTAGCGTTTTGGGGCTAGTGAGATTGTGGGCGATGGGTTGAAAGTATCTGGCAGACATGTTAGTA  +1,+1

WT gRNA2 ATAGTTTGTGTGGATTACCCTAG·ACCACAG·ATAGATGATACAGTTAATTTCATTGAAGCAGCTTCCTTATCTGCTAGTTTT
P  (1)   ATAGTTTGTGTGGATTACTCTAG·ACCACAT·ATAGATGATACAGTTAATTTCATTGAAGCAGCTTCCTTATCTGCTAGTTTT  S2
P  (1)   ATAGTTTGTGTGGATTACCCTAGCACCACAG·ATAGATGATACAGTTAATTTCATTGAAGCAGCTTCCTTATCTGCTAGTTTT  +1
P  (1)   ATAGTTTGTGTGGATTACCCTAG·ACCACAG·AAAGATGATACAGTTAATTTCATCGAAGCAGCTTCCTTATCTGCTAGTTTT  S2
P  (1)   ATAGTTTGTGTGGATTACCCTAC·ACCACAG·ATAGATGATATGGTTAATTTCATTGAAGCAGCTTCCTTATCTGCTAGTTTT  S3
44 (4)   ATAGTTTGTGTGGATTACCCTAG·ACCACAG·ATAGATGATACGGTTAATTTCATTGAAGCAGCTTCCTTATCTGCTAGTTTT  S1
44 (8)   ATAGTTTGGGTGGATTACCCTAC·ACCACAG·ATAGATGATACAGTTAATTTCATTGAAGCAGCTTCCTTATCTGCTAGTTTT  S2
45 (2)   ATAGTTTGTGTGGATTACCCTAG·ACCACTCCATAGATGATACAGTTAATTTCCTTGAAGCAGCTTCCTTATCTGCAAGTTTT  +1,S4
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Figure 3. Targeted mutations detected in melon plants and protoplasts induced by CRISPR/Cas9 in each gRNA. Target sequences of *CmPDS* are bolded and the protospacer adjacent motif (PAM) in italics. Aligned sequence data is shown for four plants (number) and protoplasts (P). Number of colonies sequenced are indicated in the left between parentheses. Substitutions are highlighted in yellow; insertions are highlighted in blue, whereas a · is used in sequences with no insertion to maintain the reading frame; and, deletions are highlighted in red.

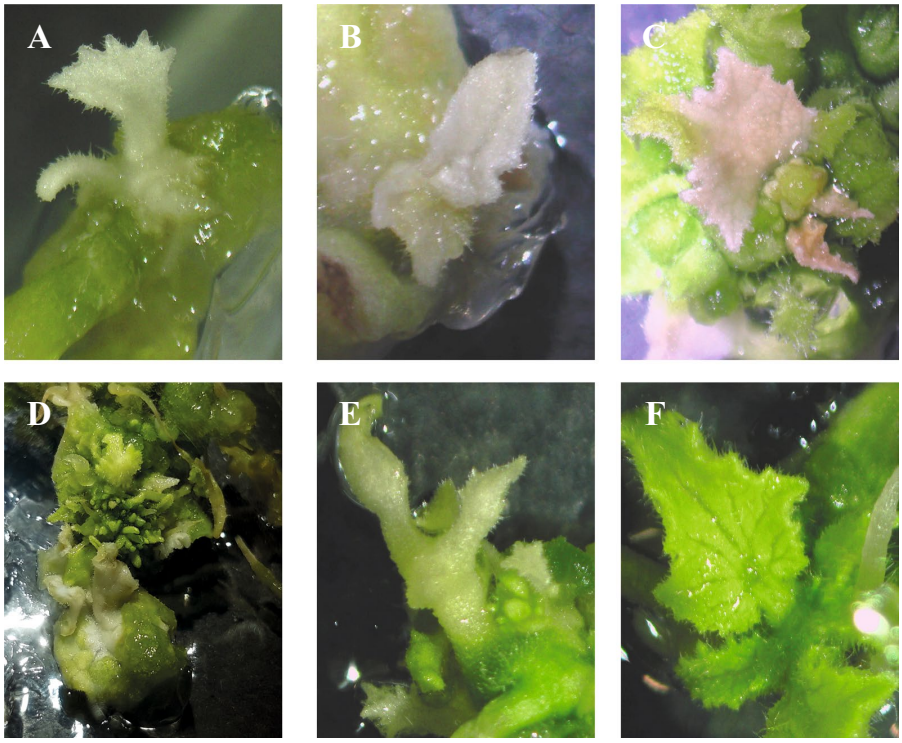

Figure 2. Phenotypic diversity of regenerated CRISPR/Cas9 mutated plants. (A,B) panels show fully albino and dwarf melon plants; (C–E) panels show chimeric albino plant presenting a mixture of green and white tissues; and (F) panel, shows a green WT regenerated plant.

Targeted mutagenesis in transgenic melon plants. Polymerase chain reaction (PCR) was performed in genomic DNA of the regenerated plants to detect the presence of the transgene and to amplify the target gene sequences. Genomic DNA was extracted from 62 plants. A 239 bp fragment within the LB and RB regions was amplified to confirm the presence of transgene. The transformation efficiency on the regenerated plants was 71%. A 502 bp fragment of the *CmPDS* gene corresponding to the first and second exons together was amplified, and a mutation analysis was performed with 10 plants showing a clearly *PDS*-edited phenotype (Fig. 3). A variety of mutations was detected in the genotyped plants, including insertions, deletions and substitutions (Fig. 3 and Table S3). In 113 sequenced colonies from albino phenotype plants analyzing both gRNAs, the target mutation

efficiency was 45% and 42% for gRNA1 and gRNA2, respectively. Up to 91% of mutations were substitution events, 7% were insertions, and 2% deletions. Further analysis of the *CmPDS* gene mutations revealed substitutions, insertions and deletion events upstream and downstream of the target region for both gRNAs. Multiple alleles were found in all albino and chimeric plants, showing a high level of chimerism for both target sequences (Table S3). *In silico* analysis of codon change due to substitutions in plants revealed that 28.56% of substitutions caused an introduction of a STOP codon, 60.71% caused a change in amino acid (AA) codon, and a 10.71% caused no AA codon change.

Discussion

To date, CRISPR/Cas9 system has been applied for basic research and trait development in many plant species[19,20]. To our knowledge, this study reports for the first time gene editing by CRISPR/Cas9 in melon (*Cucumis melo*). We employed this system to target the *PDS* gene in melon, a key enzyme in the carotenoid biosynthesis pathway involved in at least 20 metabolic pathways, including the inhibition of many genes in carotenoid, chlorophyll, and GA biosynthesis pathways[21]. Successful disruption of *PDS* gene results in a generation of mutants expected to be photobleached or albino, which allows to phenotypically identify the feasibility of CRISPR/Cas9 in melon. Alteration of *PDS* was chosen as a visual marker to easily detect the CRISPR/Cas9 genome editing in melon. In order to improve our chances of success we designed two gRNAs and adapted an existing genetic transformation system in cucumber[22]. Chimeric plants, showing albino and green tissue in the same plant or leaf, and albino plants were regenerated from melon cotyledonary explants, as reported in many other species[23–27]. A total of 4 fully albino plants and 13 albino chimeric plants were regenerated due to the loss-of-function of *CmPDS* gene.

Most of the mutations analyzed in melon protoplasts were substitutions and two insertions were found. Mutations in transgenic melon plants were mostly substitutions, with some deletion and insertion events. Although INDELs are the most common mutations induced by NHEJ, other authors have found similar results concerning the high level of substitutions induced by CRISPR/Cas9. For example, in soybean protoplasts and plants, a high level of substitutions were analyzed in the mutations events[28]; in protoplasts of cotton, only substitutions were detected[29]; in cassava plants, substitutions occurred more frequently than INDELs mutations[24]; and, in rice, the 25–45% of the analyzed mutations were substitutions[30].

Agrobacterium-mediated transformation is the only reported method for genetic transformation in melon. One of the major issues when transforming species of the *Cucurbitacea* family is the occurrence of "escapes", which are non-transgenic well-developed shoots regenerated into selective media. In melon, the regeneration rates from cotyledonary explants are usually high (40–70%), nevertheless, transformation rates of the regenerated shoots are usually very low, and is highly genotypic dependent[22,31]. Melon, of the *Cucurbitacea* family, is a recalcitrant crop for transformation[31]. In our study, 8% of the cotyledonary explants regenerated shoots, among which a high percentage (71%) proved to be transgenics. Although we did not find biallelic mutations in melon plants and albino plants failed to grow *in vitro*, mutations should be stably inherited in the T1 and T2 generations[32]. The lethal phenotype of PDS mutant plants could be alleviated by optimizing the medium. The use of an additional medium to grow albino plants, increasing sugar composition or increasing cytokinin proportions could allow the survival of albino plants. Furthermore, plant regeneration from cotyledonary explants can induce endoduplication phenomenon that generate tetraploid regenerants[33]. In this experiment we did not analyze the ploidy of the regenerated albino plants because majority of albino plants were also dwarf hence plant material availability was low.

The pHSE-CmelPDS vector contained the Cas9, a gRNA scaffold and both gRNA guides. Promoters from the cauliflower mosaic virus (CaMV35S) and *Arabidopsis thaliana* (U6 and U3), were used for driving the constitutive expression of Cas9 and both sgRNA (gRNA1 and gRNA2), respectively. Within dicotyledons, when CaMV35S promotor is used the mutation rate depends on the species, from 20 to 100%[26,34]. High transcription levels of Cas9 protein and sgRNA are essential for the activity of the CRISPR/Cas9 system[35]. The expression driver of Cas9 gene is an important factor that can increase the mutation frequency, and the use of a endogenous promoter ensures a high level of transgene expression and allows to increase mutation efficiencies 2 to 7 fold, as showed in soybean and liverwort[28,36]. The endogenous U6 promoter for sgRNA expression has a key role in the CRISPR/Cas9 efficiency too[37]. The regeneration efficiency of mutant plants from cotyledonary explants (1.8%) was very similar to those reported in watermelon[12]. The mutation rate of the sequenced colonies was 42–45% in the analyzed transgenic plants, which is lower than the ones found in other dicotyledons. In protoplast transfected cells, mutation was successful despite low edition frequencies, 25% for each gRNA. In comparison to other species this value is low and could be due either to low transfection efficiency or to a lack of CRISPR/Cas9 editing activity. It is possible that the fact that the promoters used were not endogenous is the cause of the efficiency drop of the CRISPR/Cas9 system. Overall, the use of endogenous promoters for Cas9 and sgRNA expression is the best way to increase the CRISPR/Cas9 efficiency in melon and other cucurbits.

Efficient gene editing in melon presents the possibility to study new gene functions for basic research, and new opportunities for melon productivity by improving biotic stress, melon production and the post-harvest utilization. Towards this, we demonstrated the occurrence of targeted mutagenesis with the CRISPR/Cas9 system in melon protoplasts and plants. The *CmPDS* knockout system described generates easily detectable albino and dwarf plants and mutation events in a frame of 6–8 weeks. Therefore, it provides a valuable method to facilitate rapid assessment and optimization of CRISPR/Cas9 and other genome-editing technologies in melon.

Materials and Methods

Vector construction. Construct for constitutive expression of Cas9 was done following the protocol of Xing *et al.*[38]. The binary vectors pHSE401 and pCBC-DT1T2 (Addgene plasmids #62201 and #50590, respectively) were a gift from Qi-Jun Chen. For the assembly of two gRNAs into pHSE401, a four-primer mixture with DT1-F0-PDS/DT2-R0-PDS and DT1-BsF-PDS/DT2-BsR-PDS in a proportion 1:20 (Table S5), were used for PCR

amplification along with pCBC-DT1T2 and Phusion High-Fidelity DNA Polymerase (NEB) following the manufacturer's recommendations. PCR protocol was 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min and a final extension at 68 °C for 5 min. PCR product DT1T2-PCR (626 bp) was separated on 2% agarose gel and agarose purified with PureLink Quick Gel Extraction Kit (Invitrogen). Then, DT1T2-PCR product was assembled into pHSE401 by Golden Gate cloning method, using *Bsa*I and T4 Ligase (NEB) following the manufacturer's recommendations. The binary vector constructed, named as pHSE-CmelPDS (Fig. 1C), containing both guides, gRNA1 and gRNA2, was used to transform NEB 5-alpha Competent *E. coli* (High Efficiency; NEB). Positive clones were confirmed by Sanger sequencing, plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and finally transformed into *Agrobacterium tumefaciens* EHA105.

Extraction of melon protoplast and its transformation. Melon protoplasts were extracted and transfected following the protocol from Yoo *et al.*[39] with minor modifications. 10–15-days old melon cotyledons were chopped with a razor blade and digested with 10 mL of enzyme solution (20 mM MES, 1.5% cellulase R10, 0.4% macerozyme R10, 0.4 M mannitol, 20 mM KCl and pH 5.7) and incubated in an orbital shaker for 3 h at 24 °C and 70 rpm. Then, the protoplast solution was collected on ice into a 15 mL tube and diluted up to 10 mL with W5 solution (2 mM MES, 0.5 M mannitol, 20 mM KCl and pH 5.7). The protoplast solution was filtered through a 75-µm mesh, and protoplasts were collected by centrifuging at 100 g for 5 min at 4 °C. The supernatant was discarded and protoplasts were resuspended up to 10 mL of W5. Protoplasts were kept on ice for 30 min, the supernatant was discarded, and the protoplast solution was adjusted to a concentration of $2{\cdot}10^5$ protoplasts·mL^{-1} with the help of a hemocytometer with MMG solution (4 mM MES, 0.4 mannitol, 15 mM $MgCl_2$ and pH 5.7). For the transformation, 10 µL of pHSE-CmelPDS vector (20–30 µg) were mixed with 100 µL of protoplasts ($2{\cdot}10^4$) and 110 µL of PEG-calcium transfection solution (40% PEG4000, 0.2 M mannitol, and 100 mM $CaCl_2$), and incubated for 15 min at 25 °C in darkness. Transfection was stopped by adding 400 µL of W5 supplemented with 5 mg·L^{-1} hygromycin and cultured in darkness for 24 h at 24 °C. The transfected protoplasts were then collected for genomic DNA extraction.

Agrobacterium-mediated transformation and plant regeneration. *Cucumis melo* var. *cantaloupensis* inbred line "Charentais" (provided by ROCALBA S.A.) was used as the source of explants for genetic transformation. Seed coats were removed, and seeds were surface-sterilized with a 20% sodium hypochlorite solution containing 8 drops·L^{-1} of Tween 20 for 10 min in agitation and rinsed two times with sterile distilled water. Seedlings were pre-cultured on MS medium supplemented with 3% sucrose for two days. Then, nodal cotyledons were used as explants after removing the embryo. Explants were vacuum-infiltrated with *Agrobacterium tumefaciens* EHA105 infection solution for 5 min twice and an additional 5 min in a shaker without vacuum. To prepare the *Agrobacterium tumefaciens* EHA105 infection solution, a single colony containing the selected binary CRISPR/Cas9 vector was picked and put in a 5 mL starter culture of LB containing 50 mg·L^{-1} Kanamycin and 100 mg·L^{-1} Rifampicin and cultured for 20 h at 24 °C at 130 rpm. Next, 1 mL of the starter culture was transferred to 45 mL LB culture containing Kanamycin and Rifampicin and cultured until OD_{600} reached 0.6. Once the *Agrobacterium* transformation was carried out, explants were transferred to co-cultivation medium consisting on MS medium supplemented with 3% sucrose and cultured 48 h at 24 °C in the dark. Afterward, explants were rinsed with antibiotic solution composed of 500 mg·L^{-1} Cefotaxime and 300 mg·L^{-1} Ticarcillin in distilled water. Then, the explants were transferred into selective medium which was MS supplemented with Sucrose 3%, 2.22 µM BAP, 0.48 µM IAA, 4 µM $CuSO_4{\cdot}5H_2O$, 500 mg·L^{-1} Cefotaxime, 300 mg·L^{-1} Ticarcillin and 5 mg·L^{-1} hygromycin B. Every two weeks explants were transferred to fresh selective media. Regenerated plants were transferred to E20A[40] media supplemented with 5 mg·L^{-1} hygromycin B. Explants on selective medium and plants were cultured under a 16/8 h light/dark photoperiod at 24 °C.

Detection of transgene and CRISPR/Cas9 mutation. Genomic DNA of protoplasts and plants was extracted following the methodology of Doyle and Doyle[41] with minor modifications. The transgene presence in plant (Fig. 1B) was confirmed by PCR using specific primers named pHSE401.SeqF and pHSE401.SeqR (Table S5). A fragment flanking gRNA1 and gRNA2 (846 bp) of the *CmPDS* gene from transgenic plants and transformed protoplasts was amplified using F-CmPDS and R-CmPDS primers, and the amplified PCR product was gel-purified and cloned into pCR-Blunt II-TOPO vector (Life Technologies). Colonies were Sanger-sequenced using M13F and M13R primers for detecting specific additions, deletions and substitutions. Mutation rate was calculated as the ratio of mutated clonal colonies versus total sequenced colonies.

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Author contributions

I.H. designed the experiment, obtained the gene edited plants, interpreted the sequencing data and wrote the manuscript. C.L.-C. obtained the sequencing data and edited the manuscript. S.N. supervised the experiment.

Competing interests

The authors declare no competing interests.

Additional information

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CHAPTER 3. Chromosome doubling methods in doubled haploid and haploid inducer-mediated genome-editing systems in major crops

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REVIEW

Chromosome doubling methods in doubled haploid and haploid inducer-mediated genome-editing systems in major crops

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Abstract
The doubled haploid technique aims to generate pure inbred lines for basic research and as commercial cultivars. The doubled haploid technique first generates haploid plants and is followed by chromosome doubling, which can be separated in time or overlapped, depending the procedure for each species. For a long time, much effort has been focused on haploid production via androgenesis, gynogenesis, or parthenogenesis. The obtention of haploid plants has frequently required more optimization and has lagged behind research and improvements in chromosome doubling methods. Nevertheless, chromosome doubling has recently been of renewed interest to increase the rates and efficiency of doubled haploid plant production through trialing and optimizing of different procedures. New antimitotic compounds and application methods are being studied to ensure the success of chromosome doubling once haploid material has been regenerated. Moreover, a haploid inducer-mediated CRISPR/Cas9 genome-editing system is a breakthrough method in the production of haploid plant material and could be of great importance for species where traditional haploid regeneration methods have not been successful, or for recalcitrant species. In all cases, the new deployment of this system will demand a suitable chromosome doubling protocol. In this review, we explore the existing doubled haploid and chromosome doubling methods to identify opportunities to enhance the breeding process in major crops.

Keywords Doubled haploid · Androgenesis · Gynogenesis · Parthenogenesis · Haploid inducer · CRISPR/Cas9 · Chromosome doubling · Antimitotic

Introduction

Plant breeding has attempted over many decades to increase crop yield and improve cultivar traits. The purpose of plant breeding is to boost agronomical traits such as: disease and insect resistance; tolerance to abiotic stresses like drought, extremes of temperature and salinity; and to increase yield while at the same time, enhancing or maintaining nutritional quality.

Conventional breeding by backcrossing is a method to improve an elite line by adding a new trait. By crossing the elite line with a donor line, carrying the trait of interest, an F1 hybrid is obtained and backcrossed with the elite line again. The subsequent offspring is recurrently backcrossed with the elite line again, until the 5th–8th generations. The final backcrossed line contains the new trait of interest and the characteristics of the elite line, and the genotypic background of the donor line has been cleared (Forster et al. 2007). Backcrossing is effective, but obtaining a stabilized line with the trait of interest is extremely time consuming due to the number of generations needed to be crossed and evaluated (Fig. 1). Instead, the emergence of doubled haploid (DH) technology in the second half of the twentieth century has dramatically reduced the time required to generate pure homozygous lines.

Doubled haploid lines are pure and genetically homozygous individuals produced when spontaneous or induced chromosome duplication of haploid cells occurs. DHs are one of the leading achievements in plant breeding because

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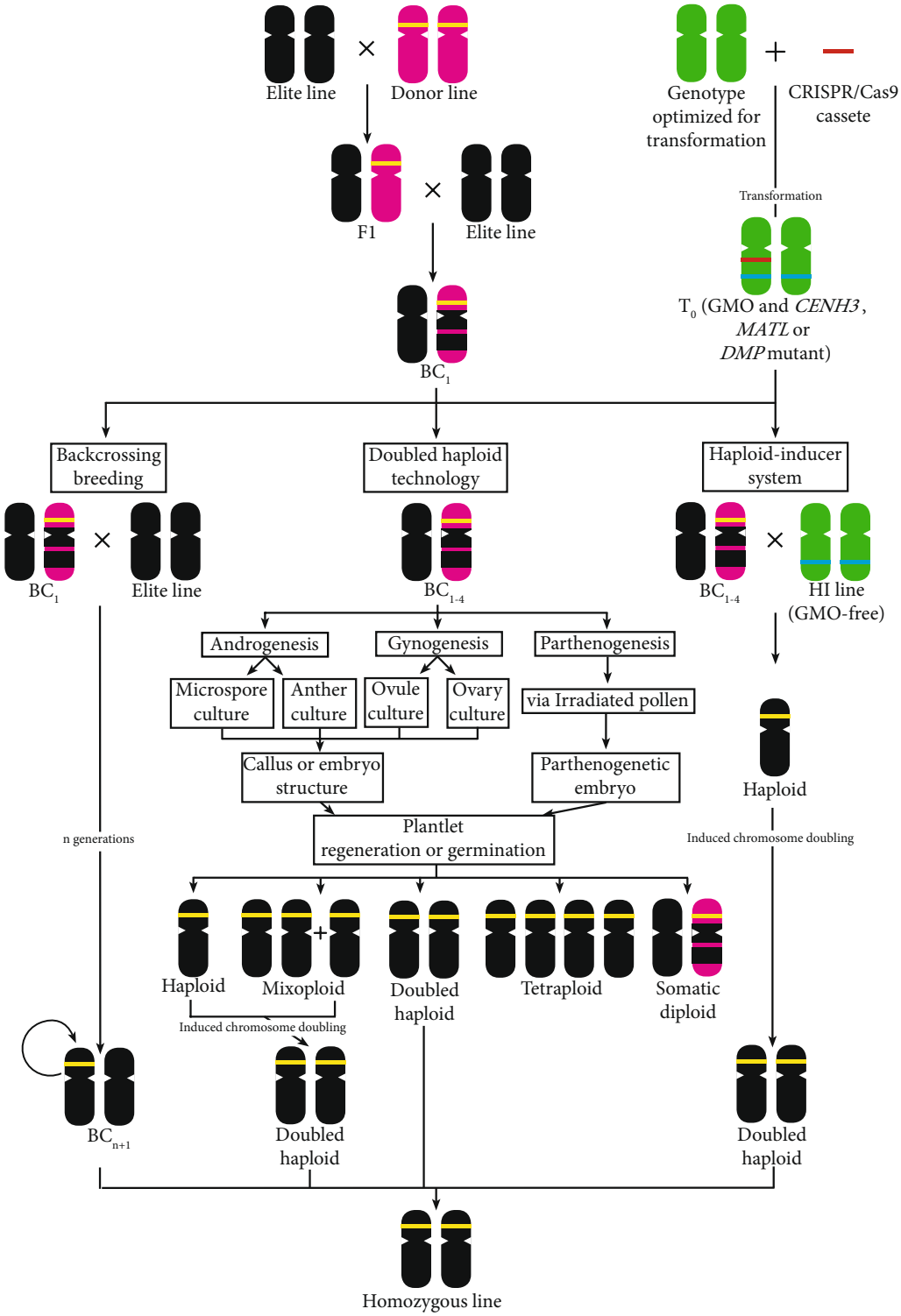
Elite line
Donor line
Genotype optimized for transformation
CRISPR/Cas9 cassete
F1
Elite line
Transformation
BC1
T0 (GMO and CENH3, MATL or DMP mutant)
Backcrossing breeding
Doubled haploid technology
Haploid-inducer system
BC1
Elite line
BC1-4
BC1-4
HI line (GMO-free)
Androgenesis
Gynogenesis
Parthenogenesis
Microspore culture
Anther culture
Ovule culture
Ovary culture
via Irradiated pollen
Callus or embryo structure
Parthenogenetic embryo
Haploid
n generations
Plantlet regeneration or germination
Induced chromosome doubling
Haploid
Mixoploid
Doubled haploid
Tetraploid
Somatic diploid
Induced chromosome doubling
BCn+1
Doubled haploid
Doubled haploid
Homozygous line

◂**Fig. 1** Schematic representation of three possible methods for producing purely homozygous lines: backcrossing breeding, doubled haploid technology and the HI-mediated CRISPR/Cas9 genome-editing system. Chromosome representation shows the genetic ploidy level according to number (one chromosome for haploids and two chromosomes for diploids) and the genetic background according to the color (black for elite receptor lines, pink for donor lines, green for genotypes optimized by genome transformation, yellow for the allele of interest, red for the CRISPR/Cas9 cassette targeting the *CENH3*, *MATL* or *DMP* gene and blue for the mutated *CENH3*, *MATL* or *DMP* gene). Backcrossing breeding can take from six to eight generations depending on the species and possible coupled to marker-assisted selection. DH technology can take from 6 months to 2 years depending on the species. An HI-mediated genome-editing system can take 1 year. DH technology and HI-mediated genome-editing system can start with BC_1 to BC_4 plants (color figure online)

completely homozygous plants can be produced within a year. DH production includes two major steps: haploid induction and chromosome doubling. Haploid induction attempts to regenerate haploid or spontaneous DH plants, which can be achieved by androgenesis, gynogenesis or parthenogenesis, depending on the species. The chromosome-doubling step is mandatory when spontaneous DHs are not regenerated and is achieved using antimitotic compounds to double the ploidy level of haploid plants.

The haploid inducer (HI)-mediated genome-editing system is a promising approach for DH production that is still under development for the majority of crops. Nowadays, genome-editing technologies are of major importance in many research areas, plant breeding included. Since the discovery of CRISPR/Cas9 in 2012, and its first use in plants in 2013 (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013), many applications of genome-editing technology have been boosted thanks to the efficiency and versatility of CRISPR/Cas9 in comparison to previous genome-editing technologies such as targeted induced local lesions in the genome (TILLING), zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). The CRISPR/Cas9 system is a genome-editing technology that enables an unprecedented control over the mutation process. CRISPR/Cas9 technology consists of a Cas9 nuclease, guided by a 20-nt gRNA sequence (gRNA), which induces DNA double-strand breaks (DSBs). DSBs are repaired by either non-homologous end-joining (NHEJ) or homology directed repair (HDR), generating insertion, and deletion events (INDELs) in a precise DNA target sequence. CRISPR/Cas9 has features over its predecessors that make its use and application easier and more efficient (Belhaj et al. 2015).

Many reviews have been published summarizing haploid production and the best methodologies for each species. Many crops have excellent specific reviews focused on the DH techniques for a plant family or single species like the Fabaceae (Croser et al. 2007), the Solanaceae (Seguí-Simarro et al. 2011), the Cucurbitaceae (Dong et al. 2016), bell pepper (Irikova et al. 2011), wheat (Niu et al. 2014), rice (Mishra and Rao 2016), and sorghum (Teingtham and La Borde 2017). In addition, the use of antimitotic agents for plant tissue polyploidization has been reviewed by Dhooghe et al. (2011), and the current and novel technologies for haploid induction have been reviewed by Ren et al. (2017). However, an evaluation is still needed of the best methodologies for DH production that combine HI technology with the application of antimitotic agents for chromosome doubling in major crops. Moreover, the opportunities that HI-mediated genome editing offers have not yet been reviewed for recalcitrant crop species.

In this review, we discuss the following aspects in major crop species: the methods of DH production; the availability of chromosome-doubling methods to obtain DH lines; the opportunities for HI-mediated genome-editing systems in DH technology, and, finally, we focus on the development of the DH technique, HIs, antimitotic agents, and chromosome-doubling protocols for the future of plant breeding.

Doubled haploid uses

DH lines are highly important for plant breeding due to their complete homozygosis, making qualitative and quantitative phenotypic selection more efficient. Since the very first attainment of DHs in *Brassica napus* (Thompson 1972), many publications have reported the development of DH lines in more than 250 species (Maluszynski 2003). Following the research conducted in the 70s and 80s that demonstrated the ability to generate DHs in many cereal, vegetable and horticultural crops, the focus in recent decades has been optimizing and assaying different ways to enhance DH production in each species and genotype by introducing changes in every step of the DH programs.

DHs have been of great importance for: establishing chromosome maps and whole genome sequencing in the vast majority of genetically mapped and sequenced species; bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations; and, for mapping quantitative trait loci (QTLs) (Forster et al. 2007). This usage of DHs in basic research has been extended into direct application in variety improvements. Furthermore, DHs can be used as commercial cultivars such as stabilized homozygous lines or as parental lines to produce F1 hybrid lines, avoiding classical breeding methods to obtain stabilized and non-segregant lines.

Strategies for the production of doubled haploid material

Gametic haploid cells are the initial material used to obtain DH lines. Gametes from meiotic cells allow the generation of plantlets when cultured in vitro or when pollinated with

irradiated pollen. The haploid step can be either a microspore from an anther or an ovule from an ovary depending on the species. The usual methods to induce haploids are as follows: androgenesis, gynogenesis, and parthenogenesis (Fig. 1). Plantlet regeneration from microspores or ovaries is a two-step protocol if a callus step is required prior to plantlet regeneration, or a one-step protocol if it directly induces an embryo or regenerates a plantlet. Besides, gametic cells from meiosis can be developed into haploid embryos, via parthenogenesis. Thus, a DH process always requires a gametic haploid step from which haploid or DH plantlet will be regenerated.

In vitro culture techniques for gametic cells in androgenesis and gynogenesis techniques allow the original gametophytic pathway of the gamete be redirected towards a sporophytic pathway where plantlets can be regenerated. Androgenesis is the most common method to produce DHs (Table 1). Isolated microspores or microspores contained in anthers are cultured in specific induction media to induce the formation of callus. Subsequently, these calli are cultured in regeneration media to regenerate fully formed plantlets. In most cereal species, androgenesis is the only or the best method for DH generation with a high rate of spontaneous doubling, in species such as: rice (Hooghvorst et al. 2018), oat (Kiviharju et al. 2017) and bell pepper (Keleş et al. 2015). Gynogenesis stimulates in vitro embryogenesis development of the unfertilized haploid egg cells. In this process, a two-step protocol is usually carried out to induce callus formation from the female ovules in induction medium and to regenerate plants from callus in regeneration medium. For onion (Fayos et al. 2015) or beet (Hansen et al. 1995), gynogenesis is the best method for DH production. The ploidy level of the androgenetic and gynogenetic regenerated plants can differ depending on the cell events related to spontaneous or induced chromosome doubling (see below). Haploids, doubled haploids, mixoploids, and tetraploids can be produced during the in vitro DH process (Fig. 1). In androgenesis and gynogenesis, it is desirable that the regenerated plantlets originate from microspore or ovule cells; nonetheless, somatic embryogenesis from anther or ovary tissues can take place. This process is defined as the regeneration of a whole plant from undifferentiated somatic cells in in vitro culture. The ploidy of these plantlets is diploid, and the genomic background is identical from which DHs are expected to be generated (Fig. 1). Parthenogenesis methodology allows the formation of an embryo from an egg cell without fertilization. Egg cells can be induced to develop into haploid embryos following in situ pollination with irradiated pollen, and these embryos only inherit the maternal set of chromosomes due to false fertilization. Such embryos germinate in vitro and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants. In the Cucurbitaceae, parthenogenesis is the only successful approach to obtain DH plant material (Dong et al. 2016).

Conventional DH technology has had great importance in some species to produce pure homozygous lines. Nonetheless, in all reported species, there is a high genotypic dependency on the efficiency of the method, with some cultivars adapted to the existing protocols and some others very recalcitrant to the process. Publications on rice, wheat, and maize show correlation with the substantial progress in haploid technology, attainable given the intensive research efforts (Croser et al. 2007). The majority of crops have acceptable DH protocols from which DH lines are produced successfully, but they require a significant time investment that ranges from 5 months to 2 years, substantial personnel and equipment needs, and always have inevitable variability in efficiency, depending on the genotype used. There are even some crops of great economic importance, including tomato species (Seguí-Simarro et al. 2011) and members of the Cucurbitaceae and Fabaceae family (Croser et al. 2007; Dong et al. 2016), that lack a successful beginning to end protocol for DH production.

Approaches and process of the chromosome-doubling step

Every DH program starts with the haploid gametophytic phase to efficiently obtain DH plants. During the latter part of the in vitro process, haploid plant material needs to undergo chromosome duplication to finally obtain a fertile plant, from which DH seeds are recovered. The original chromosome set, whether maternal (gynogenesis and parthenogenesis) or paternal (androgenesis), must go through a spontaneous or induced duplication. The effective duplication of the haploid material is essential for the success of the DH process, because haploid plants are infertile. The chromosome doubling can be spontaneous or induced. Earlier duplication is the ideal for avoiding mixoploid plants, or fully haploid plants, and to ensure a battery of DH plants.

Endomitosis and nuclear fusion are the main causes of spontaneous duplication. These processes have been extensively studied in barley and inferred in other species (Kasha 2005). During mitosis, chromosome multiplication and separation of cells usually occurs. Instead, in endomitosis, multiplication occurs but the cell fails to divide and one nucleus with two sets of chromosomes is restored. During nuclear fusion, two or more synchronized nuclei divide and develop a common spindle. Spontaneous chromosome doubling capacity during the process depends on the species and genotype. For example, the frequency of spontaneous DH androgenic bell pepper plants is 30–55% (Irikova et al. 2011; Keleş et al. 2015); in rice, it ranges between 8 and 30% of the regenerated plants (Alemanno and Guiderdoni 1994;

Table 1 Overview of the most commonly used methods for doubled haploid production and chromosome doubling and their efficiency in major crops

Species	Common name	Doubled haploid method	Induced chromosome doubling method					Chromosome doubling efficiency[a]		References
			Stage	Application	Antimitotic compound	Concentration	Exposure time	Spontaneous	Induced	
Cereal crops										
Avena sativa	Oat	Anther culture	In vitro plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	37.42%	88.17%	Kiviharju et al. (2017)
Hordeum vulgare	Barley	Microspore culture	–	–	–	–	–	> 90%	88.3–93.5%	Li and Devaux, (2003)
Hordeum vulgare	Barley	Anther culture	In vivo plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	–	–	Jacquard et al. (2003)
Sorghum bicolor	Sorghum	Anther culture	–	–	–	–	–	95.3%	–	Kumaravadivel and Sree (1994)
Secale cereale	Rye	Anther culture	–	–	–	–	–	13–67%	–	Tenhola-Roininen et al. (2005)
Triticum aestivum	Wheat	Microspore culture	Tillers of in vivo plants	Immersion	Colchicine	0.10%	5–8 h	–	95.60%	Niu et al. (2014)
Triticum aestivum	Wheat	Microspore culture	Internode of pollinated spikes	Injection	Colchicine	1% suppl. 100 ppm 2,4-D	48 and 72 h	0	33–100%	Sood et al. (2003)
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	250 $mg{\cdot}L^{-1}$	48 h	31%	65.50%	Alemanno and Guiderdoni (1994)
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	300 $mg{\cdot}L^{-1}$	48 h	0–0.18 DH green plantlets per 100 anthers	0.75 DH green plantlet per 100 anthers	Hooghvorst et al. (2018)
Oryza sativa	Rice	Anther culture	In vitro plantlets	Immersion	Colchicine	500 $mg{\cdot}L^{-1}$	5 h	–	35%	Hooghvorst et al. (2020a)
Triticum x *Rye*	Triticale	Microspore culture	Embryos	Microspore culture	Colchicine	0.3 mM	24 h	30%	50–55%	Würschum et al. (2012)
Zea mays	Maize	Anther culture	Anthers	Induction medium	Colchicine	0.2 $g{\cdot}L^{-1}$	3 d	19%	20%	Obert and Barnabás (2004)
Zea mays	Maize	Anther culture	Microspores	Induction medium	Colchicine	1,250 µM	1 w	40%	93.75% DH plantlets for 100 anthers	Antoine-Michard and Beckert (1997)
Vegetable and horticultural crops										
Apiaceae or Umbelliferae										
Daucus carot	Carrot	Microspore culture	In vivo plants	Immersion	Colchicine	0.34%	1.5 h	50%	–	Ferrie et al. (2011)

Table 1 (continued)

Species	Common name	Doubled haploid method	Induced chromosome doubling method					Chromosome doubling efficiency[a]		References
			Stage	Application	Antimitotic compound	Concentration	Exposure time	Spontaneous	Induced	
Pastinaca sativa	Parsnip	Microspore culture	In vivo plants	Immersion	Colchicine	0.34%	1.5 h	50%	–	Ferrie et al. (2011)
Brassicaceae										
Brassica napus	Rapeseed	Microspore culture	Microspores	Induction medium	Colchicine	500 mg·L^{-1}	15 h	45–64.3%	83–91%	Zhou et al. (2002)
Brassica oleracea var. *capitata*	Cabbage	Microspore culture	Rooted in vitro plantlets	Immersion	Colchicine	0.2% suppl. 2% DMSO	9–12 h	0–76.9%	58.3–75%	Yuan et al. (2015)
Brassica oleracea var. *italica*	Broccoli	Microspore culture	Rooted in vitro plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	6–12 h	50.6–100%	54.5–58.3%	Yuan et al. (2015)
Cucurbitaceae										
Cucumis melo	Melon	Parthenogenesis	In vivo plants	Immersion	Colchicine	0.50%	2 h	–	46.03%	Solmaz et al. (2011)
Cucumis melo	Melon	Parthenogenesis	In vivo plants	Immersion	Colchicine	0.50%	2 h	23%	20.93% DH and 9.30% mixoploids	Hooghvorst et al. (2020b)
Cucumis sativa	Cucumber	Parthenogenesis	In vitro microcuttings	Culture medium	Colchicine	500 mg·L^{-1}	48 h	0%	30% DH and 55% mixoploid	Claveria et al. (2005)
Cucumis sativa	Cucumber	Parthenogenesis	In vitro nodal explants	Immersion	Oryzalin	50 mg·L^{-1}	18 h	–	86.21%	Ebrahimzadeh et al. (2018)
Solanaceae										
Capsicum annuum	Bell pepper	Anther culture	In vitro axillary buds	Lanolin paste in axillary buds	Colchicine	1%	–		n/a	Gyulai et al. (2000)
Capsicum annuum	Bell pepper	Anther culture	–	–	–	–	–	22.2–53.4%	–	Keleş et al. (2015)
Solanum melongena	Eggplant	Anther culture	In vitro axillary buds	Lanolin paste in axillary buds	Colchicine	0.50%	–	60%	25%	Corral-Martínez and Seguí-Simarro, (2012)
Solanum tuberosum	Potato	–	Nodal segment	Immersion	Oryzalin	25 µM	8 h	20–78%	10.10%	Greplová et al. (2009)

Table 1 (continued)

Species	Common name	Doubled haploid method	Induced chromosome doubling method					Chromosome doubling efficiency[a]		References
			Stage	Application	Antimitotic compound	Concentration	Exposure time	Spontaneous	Induced	
Other important plant crops										
Allium cepa	Onion	Gynogenesis	Embryos	Solid elongation medium	APM	25 µM	24 h	–	35%	Fayos et al. (2015)
Allium cepa	Onion	Gynogenesis	In vitro plantlets	Culture medium	Colchicine	10 mg·L^{-1}	3 d	1%	46%	Campion et al. (1995)
Allium cepa	Onion	Gynogenesis	In vitro *plantlets*	Media culture	Oryzalin	10 µM	3 d	–	67%	Jakše and Bohanec (2003)
Asparagus officinalis	Asparagus	Anther culture	In vitro shoot tips	Apical lanolin application	Colchicine	1.20%	–		21–97%	Tsay (1997)
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	Colchicine	0.40%	2.5 h	7.10%	4 DH per 100 ovules	Hansen et al. (1995)
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	AMP	100 µM suppl. 1.5% DMSO	5 h	6.60%	4.7 DH per 100 ovules	Hansen and Andersen (1998)
Beta vulgaris	Beet	Ovule culture	In vitro plantlets	Agarose culture medium	Trifluralin	3.4 mg·L^{-1}	36 h	0–10%	62.50%	Gürel et al. (2000)
Nicotiana tabacum	Tobacco	Anther culture	In vivo plant	Root dipping	Colchicine	0.50%	24 h		21–32%	Sood et al. (2013)

h hours, *d* days, *w* weeks

[a]If not specified, percentages refer to chromosome doubled material relative to the initially treated material

Hooghvorst et al. 2018; López-Cristoffanini et al. 2018). In species whose spontaneous doubling rate is high, the process of induced chromosome doubling has not been explored to any great extent for obvious reasons. Nevertheless, despite this, some genotypes are more likely to regenerate spontaneous DHs than others, because all in vitro processes are genotypically dependent, including duplication. Indeed, species with generally high rates of spontaneous DH still need efficient protocols to induce chromosome doubling with antimitotic compounds, because some genotypes within such species have low rates of spontaneous DH generation or no spontaneous production at all, meaning that antimitotic application is still essential.

A proportion of the so-called 'spontaneous' duplication reported in the literature is actually induced via chromosome doubling by means of pre-treatments like heat or cold pre-treatments that do not involve antimitotic. Temperature stress, like heat or cold pre-treatments, is usually applied during androgenesis and gynogenesis prior to in vitro culture. Many of these pre-treatments were originally intended to increase microspore induction, but they usually increase the frequency of chromosome doubling due to the destabilization of microtubules and microfilament elements that form the cytoskeleton (Kasha 2005). In microspores, cold pre-treatment is related to failure of cell wall formation leading to multinucleate structures [known as coenocytic structures, see Testillano et al. (2002)] which result from nuclear division without cytokinesis. However, in spite of the basic research that has related pre-treatment protocols to increased number of microspores at the optimal stage, once the microspore culture is started and plants regenerate, it is difficult to demonstrate that the pre-treatment is the causal agent of the increase in frequency of DHs beyond the determination of the best microspore stage for embryogenesis.

Induced chromosome duplication may be feasible at different stages of the process: at the first pollen mitotic division of microspore cells, at the callus stage when growing in vitro, or at the plant stage when regenerated (Table 1). The use of antimitotic compounds is mandatory when spontaneous chromosome doubling is absent or very low. A specific type of endomitosis, known as C-mitosis, takes place when antimitotic compounds are used to destabilize the cell cycle, perturbing not only mitosis but also arresting cells during interphase (Lu et al. 2012). In interphase, DNA is replicated and each replicated chromosome forms sister chromatids that are bound by the centromere's spindle tubules. When C-mitosis occurs, the antimitotic compound interacts with tubulin subunits destabilizing and inhibiting their assembly. Antimitotic treatment depends on the species and the protocol used for obtaining DH plants. Some key features considered are the antimitotic agent, its concentration, the exposure time and the treatment stage, which were thoroughly reviewed by Dhooghe et al. (2009).

In androgenetic protocols for DH production, free-microspores or microspores contained in anthers are usually cultured in colchicine-supplemented medium. By this means, chromosome doubling is achieved at earlier stage ensuring the success of the process. In addition, an increased level of microspore induction has been described attributed to the presence of colchicine (Alemanno and Guiderdoni 1994; Iqbal et al. 1994; Barnabás et al. 1999; Hooghvorst et al. 2018).

Haploid plants as explant material are another important source for recovering DHs. Most of the DH protocols apply antimitotic compounds to plants grown either in vitro or in vivo to achieve chromosome doubling (Table 1). Immersion of the whole in vitro plantlet and the apical meristem in vivo are two approaches that usually yield good chromosome doubling results in many plant species (Table 1). In vitro treatments usually take longer and the antimitotic concentration is lower, whereas in vivo treatments have shorter exposure times with higher concentrations of compound. Nevertheless, in some species like onion, the apical meristem of adult plants is inaccessible, impeding chromosome doubling in vivo (Bohanec 2002). Meanwhile, during propagation of regenerated haploid plants, chromosome doubling has also been achieved by adding antimitotic compounds to the culture medium. Breeders sometimes discard androgenetic and gynogenetic haploid plants when spontaneous doubled regenerants are considered acceptable. Despite their great potential, the lack of a successful protocol for chromosome doubling in grown plants means that they frequently go to waste. In contrast, the parthenogenetic process usually depends on whole plants or embryos as the source of material for DH (Table 1).

Several antimitotic agents have been used for chromosome doubling of haploid plants. Colchicine is the most popular antimitotic agent used for DH studies in most species because it has a high chromosome doubling ability. Yet there are many different chemicals with antimitotic effects such as amiprophosmethyl (AMP), pronamide, propham, oryzalin and trifluralin, which have similar effect and mechanisms of action to colchicine (Bartels and Hilton 1973; Lu et al. 2012). Colchicine is a toxic natural product extracted from plants of the *Colchicum* genus and used as an antimitotic agent. It is known to inhibit mitosis in a wide variety of plant and animal cells by interfering with the structure of the mitotic spindle (Eigsti and Dustin 1955). Furthermore, research shows that the colchicine-binding protein is a subunit of microtubules. It has been reported that low dosages of the compound can effectively halt cell division for a small period of time, thus producing a doubling of the genetic load in some cells (Borisy and Taylor 1967). Nevertheless, colchicine has a highly toxic effect on plant and animal cells, being a hazardous compound for researchers to use in the laboratory. Moreover, when plants are treated with colchicine to induce doubling, the mortality

rate is usually high, and is dependent on the concentration, time of exposure and species. Consequently, to optimize protocols, breeders need to balance dose and exposure time to ensure successful chromosome duplication while limiting the mortality rate.

Nowadays, oryzalin and trifluralin are being widely employed as substitute for colchicine. These compounds are dinitroaniline herbicides reported to bind to plant tubulin, which in turn confers an antimitotic effect similar to colchicine. Unlike colchicine, dinitroanilines have no effect on microtubules in vertebrate cells, which are resistant to its depolymerizing effects. It has been demonstrated that oryzalin has a high-affinity interaction with plant tubulin, binding rapidly and reversibly while forming a tubulin–oryzalin complex (Hugdahl and Morejohn 1993). The properties of dinitroaniline binding to tubulin are different from colchicine. Dinitroaniline binding is time independent and the tubulin–dinitroaniline complex dissociates completely, unlike colchicine, which has been reported to bind slowly to tubulin and the tubulin–colchicine complex does not easily dissociate. Furthermore, oryzalin has been reported to have a much higher affinity for unpolymerized tubulin than the polymerized form (Borisy and Taylor 1967; Hugdahl and Morejohn 1993). Scientific interest lies in the fact that dinitroanilines can be much less hazardous for humans than colchicine and equally effective at lower doses.

Key features commented above for a successful chromosome doubling need to be determined empirically for each species. More work is required concerning the chromosome-doubling step in many species and genotypes, describing the best results of different treatments (Table 1).

The antimitotic compound is usually dissolved in dimethyl sulfoxide (DMSO). DMSO has a double utility, as a solvent to dissolve the antimitotic and to increase cell permeability by allowing an increase in absorption of the agent into the plant (Hamill et al. 1992). However, DMSO may increase plant mortality due to its relative toxicity (Dhooghe et al. 2011). Other solvents can be used instead, such as NaOH or 70% ethanol for oryzalin, acetone for trifluralin, or even water (Dhooghe et al. 2011). If chromosome doubling is performed by immersion of some part of the plant in an antimitotic solution, detergent or surfactants can be added to enhance the surface contact. In contrast, if the treatment is applied to a specific area on the plant, such as the lateral or apical meristems, lanolin paste can be used to localize a dose of solution.

CRISPR/Cas9: a new actor in DH technology

The haploid induction strategy is based on intraspecific crossing to obtain haploid progeny through an HI line. HI lines have the ability to produce haploid embryos upon pollination of a receptor line. Due to a mutation in a specific gene, which is essential for the normal fertilization of female gametic cells, fertilization is impeded, and egg cells develop into haploid embryos. Natural HI lines have been used since the beginning of modern breeding in maize (Coe 1959), barley (Kasha and Kao 1970), tobacco (Burk et al. 1979), and wheat (Laurie and Bennett 1988). For instance, in maize, HI lines were discovered to carry a 4-bp insertion in the carboxy terminus of the MATRILINEAL (*MATL*) gene, also known as NOT LIKE DAD (*NLD*) (Gilles et al. 2017) or PHOSPHOLIPASE A1 (*PLA1*) (Liu et al. 2017), which encodes a pollen-specific phospholipase determined as the causal factor in the haploid induction process (Prigge et al. 2012). Phospholipase are essential enzymes expressed during pollen development and play a critical role in pollen development and germination and tube growth, its mutations are associated with delayed pollen germination and tube growth (Kim et al. 2011). Nevertheless, few species have natural HI lines; the production of HI lines in the laboratory through genome-editing techniques is a major challenge in haploid technology to improve the DH process (Fig. 1). As previously mentioned, TILLING, ZFNs, and TALENs are potential genome-editing tools to produce positive mutants. Despite this, the random mutations of TILLING, and the complex, time-consuming engineering and unwanted off-target mutations of ZFNs and TALENs have meant that the CRISPR/Cas9 system has become genome-editing system of choice.

DH technology has experimented a substantial progress resulting from the advances in engineered HI lines. Recently, *MATL* has become a target gene for genome editing in rice and maize with the CRISPR/Cas9 system, taking advantage of gene mutation during haploid induction (Table 2). In maize, maternal haploids were obtained with an efficiency of ca. 6.7% using the HI technique (Kelliher et al. 2017) and in rice, the average haploid induction rate was ca. ~ 6% (Yao et al. 2018). In wheat, a difficult species to work with because of their polyploidy, a 18.9% of haploid progeny was obtained using *MATL*-edited plants (Liu et al. 2019). On the other hand, another promising gene for HI, *DMP* (DOMAIN OF UNKNOWN FUNCTION 679 membrane protein), was demonstrated to induce haploids with a rate of 0.1–0.3% in maize. *DMP* is a pollen-expressed gene highly expressed during the late stage of pollen development and localized to the plasma membrane (Zhong et al. 2019). Despite of the lower rate of HI due to the loss-of-function of *DMP* gene, the combination of *ZmDMP* and *ZmPLA1* (or so-called *MATL* or *NLD*) genes knockout in the CAU6$^{(zmpla1-zmdmp)}$ line triggered and enhanced HI rate by five- to sixfold (Zhong et al. 2019) (Table 2). These rates of haploid production represent a breakthrough point that has the potential for further improvements. This new DH-generation pathway

Table 2 Summary of the haploid inducer-mediated genome-editing systems using CRISPR/Cas9

Species	Common name	Target gene	Haploid induction rate (%)	References
Monocotyledonous				
Triticum aestivum	Wheat	*MATL*	18.9	Liu et al. (2019)
Oryza sativa	Rice	*MATL*	2–6	Yao et al. (2018)
Zea mays	Maize	*DMP*	0.1–0.3	Zhong et al. (2019)
Dicotyledonous				
Arabidopsis thaliana	Arabidopsis	*DMP*	2.1	Zhong et al. (2020)
Arabidopsis thaliana	Arabidopsis	*CENH3*	1.1–44	Kuppu et al. (2020)

could be implemented for other secondary cereals like barley, oat, rye or triticale because existing DH protocols are less efficient than in species like rice, maize or wheat. With many genetic transformation methods validated and whole genome sequencing available, breeders should be able to take advantage of haploid induction validated technology to increase the number of DHs and the efficiency of DH production in recalcitrant species via HI-mediated genome-editing technology, avoiding the need to test in vitro androgenic protocols for a range of genotypes.

Concerning dicotyledons, the main target for the HI technique has been the *CENH3* (centromeric histone 3) gene, which is a histone present in all plants that determines the position of the centromere and like other histones, it carries an N-terminal tail, which protrudes from the nucleosome and is a target for posttranslational modification and a C-terminal histone fold domain, which interacts with DNA and other histones to form the nucleosome. CENH3 plays a major role in chromosome segregation during mitosis, its alteration may have severe or even lethal consequences (Britt and Kuppu 2016). Since its discovery, much research has been conducted to elucidate its function and capacity during haploid induction in dicot species. Nevertheless, the specific mechanism and functions are still not clear. In 2010, major progress was achieved when studying the function of *CENH3* in *Arabidopsis thaliana* (Ravi and Chan 2010). This work demonstrated that *GFP-tailswap* (*cenh3*-1 mutant plants) plants underwent haploid induction when crossed with a *CENH3* wild type line by eliminating chromosomes of the mutant line. Recently, the late HI-related gene discovered was *DMP* was used for haploid induction in dicots. The loss-of-function of the *DMP* gene in Arabidopsis, a *ZmDMP*-like gene, induced haploid progeny in a rate of 2.1% (Zhong et al. 2020) (Table 2). Besides, the *CENH3* gene was disrupted in maize too through *CENH3-tailswap* transgenic complementation, reaching a 3.6% of HI (Kelliher et al. 2016).

Hence, the *MATL, CENH3*, and *DMP* genes are the current targets for HI-mediated genome-editing systems in monocots and dicots. However, other genes related to chromosome segregation during mitosis, or pollen development have potential as target genes for HI technology using CRISPR/Cas9, as reviewed by Ren et al. (2017).

Parthenogenetic approaches of haploid production via HI lines obtained by CRISPR/Cas9 can increase the chances of DH plant generation and ease the usual time-consuming and labor-intensive processes of androgenesis, gynogenesis and parthenogenesis (Fig. 1). Further, the genotype dependency of many of the in vitro steps, like callus induction and plant regeneration, or in vivo steps like pollination with irradiated pollen, might be avoided using an HI approach, and this may also enable haploid plant material to be obtained from recalcitrant genotypes. To obtain *MATL*-, *CENH3*-, or *DMP*-mutated lines for use as HIs, the transformation should be optimized in at least one genotype for each species because the regeneration and mutation processes are mandatory. This HI-mutated genotype could be used to pollinate many genotypes of interest for haploid embryo generation, avoiding the need to optimize the process for recalcitrant genotypes.

The HI-mediated genome-editing approach for DH production for breeding purposes is a major discovery. Apart from improvements in the application of HIs discussed above, for a number of crop species, the HI technique might be the best, if not the only way, to produce DHs. Much research needs to be done on this aspect to confirm the ease of work in parallel with better results. If the production of haploid plants is more efficient using the HI approach, only the chromosome-doubling step at the haploid plant stage will become the bottleneck in achieving an efficient production of DH lines (Fig. 1).

Another important approach to the HI technique is the HI-Edit system, where successful one-step genome editing is achieved. Kelliher et al. (2019) crossed HI lines carrying the CRISPR/Cas9 cassette targeting genes for phenotypical evaluation with inbred lines to test the ability to produce positive genome-edited mutants in the haploid offspring. The intraspecific crossing in maize led to a mutant haploid descendance of 2–8%, depending on the target gene. The interspecific crossing between a wheat inbred line and a maize line homozygous for the Cas9 gene, resulted in a 1.8% rate of mutant haploids. Moreover, in Arabidopsis, 17% of the offspring were mutant haploids when crossing a HI line

with CRISPR/Cas9 system with an inbred line. Wang et al. (2019) applied the same system in maize and obtained 10 positive genome-edited plants among 245 haploids. This new system enables direct editing of elite inbred lines via a single crossing, thus overcoming recalcitrance.

Challenging families: Solanaceae, Fabaceae, and Cucurbitaceae

In plant breeding, important crops usually have a higher economical investment to increase their traits of interest. This is translated into a higher number of protocols and methodologies available for those species. DH technology follows the same pattern. However, sometimes, technical limitations related to a species or a genotype may rise difficulties to adapt a technique. It is the case of DH methodology in important families such as Solanaceae, Fabaceae and Cucurbitaceae, which despite of their economic worldwide importance, the progress achieved until nowadays is relatively low, in some cases.

It is worth noting that DH production in crop species like those from the Solanaceae could be greatly improved thanks to the HI technique. Solanaceae species are very recalcitrant to in vitro DH processes and a methodology has not been established yet for a number of species, making it difficult for breeders to use DH technology on a routine basis (Seguí-Simarro et al. 2011). For instance, DH lines can be obtained efficiently in eggplant and bell pepper through anther culture (Table 1). Nevertheless, to our knowledge, there is currently no suitable DH method available for tomato, despite all the efforts invested in DH production in this major horticultural crop. Thus, classical breeding is the only method to obtain new commercial tomato cultivars, complicating the advances of breeding selection. Therefore, generation of a HI *CENH3*-mutant tomato lines via genome editing could represent a breakthrough. This could lead to a new era for tomato breeding, avoid the current generations of self-pollination that are still required to produce inbred lines for use in hybridization. Fortunately, delivery of CRISPR/Cas9 by *Agrobacterium*-mediated transformation has been reported several times in tomato (Van Eck 2018). CRISPR/Cas9 has been successfully applied in tomato with mutation efficiencies of 80–100% for applications such as studying mutation stability of heredity in later generations (Pan et al. 2016), obtaining parthenocarpic tomato fruits (Ueta et al. 2017), and increasing plant resistance to powdery mildew (Nekrasov et al. 2017).

In the Fabaceae, attempts to produce DHs have been reported in many species (soybean, field pea, chickpea, peanut and common bean), mainly via androgenesis. Leguminous species are particularly important for low input and sustainable cropping due to their ability to fix nitrogen and as a dietary protein source for human food and animal feed (Croser et al. 2007). Nonetheless, not much progress has been made with DH technology applications, because there has been little research undertaken on these species, and the induction and regeneration rates are inherently low. Some DH lines have been produced thanks to spontaneous doubling and high rates of somatic regeneration (Croser et al. 2007; Ochatt et al. 2009). However, successful induction of chromosome doubling has not been widely reported in this family because of the scarcity of the obtained haploid material. Significantly, the CRISPR/Cas9 system has been applied in soybean multiple times (Chilcoat et al. 2017; Sun et al. 2015) and should be extended to other Fabaceae species. The HI-mediated CRISPR/Cas9 genome-editing technique presents a great opportunity to produce DHs in these species.

Another important family where HI-mediated genome-editing system could be advantageous is the Cucurbitaceae. Parthenogenesis via pollination with irradiated pollen is the best-known method to obtain haploid material among these species. However, the efficiency of the process is usually impeded by: (1) a high genotypic dependency; (2) a low production of haploid embryos; and (3) a difficulty to induce chromosome doubling of haploid plants due to mortality, hyperhidricity and a high ratio of haploids and mixoploid plants (Dong et al. 2016; Hooghvorst et al. 2020b). All this makes the process of DH production time consuming and inefficient. The HI approach in the Cucurbitaceae would be similar to parthenogenesis via irradiated pollen due to the initial pollination of the receptor plant with haploid-inducer pollen, and the rest of the process would be the same. Additional research is necessary to confirm whether the use of an HI line results in increased production of haploid embryos, reported rates lie between 0 and 5% of seeds containing haploid embryos (Hooghvorst et al. 2020b). There are a few reports of the CRISPR/Cas9 genome-editing system being applied to cucumber, watermelon and melon with mutation efficiencies ranging from 42 to 100% (Chandrasekaran et al. 2016; Hu et al. 2017; Tian et al. 2017; Hooghvorst et al. 2019a, b). On other important cucurbit species, such as *Cucurbita maxima*, *C. moschata* and *C. pepo*, there are no reports of successful generation of parthenogenetic DHs via irradiated pollen nor the application of CRISPR/Cas9 system. Nevertheless, HI-mediated genome editing should afford great opportunities for breeding in these species as well.

Despite all the other improvements in DH line production that have emerged, chromosome doubling is one step that has been inherited from the classical DH approach (Fig. 1). It is, therefore, of major importance to adapt and optimize new chromosome-doubling protocols via antimitotic compounds to increase the number of DH lines derived from the improved HI protocols. Some species, such as sorghum

and bell pepper, do not have an optimized well-described chromosome-doubling protocol because of their high level of spontaneous DH regenerants (Table 1). For instance, in rice, the spontaneous chromosome-doubling rate is usually very high, ranging from 30 to 80% (Table 1), and induced chromosome doubling has been ignored since the generation of the first DHs (Niizeki and Oono 1968). Indeed, before 2019, no reliable reports on doubling rice plants were published, authors treated androgenic haploid plants with a recovery of 35% (Hooghvorst et al. 2020a). On the other hand, species whose DH production is impeded by poor performance in tissue culture do not have a reliable described method for chromosome doubling; this is the case of rye, watermelon, other secondary cucurbit species, tomato and leguminous species (Croser et al. 2007; Forster et al. 2007; Seguí-Simarro et al. 2011; Dong et al. 2016).

HI-CRISPR/Cas9 legislative future

Another important aspect of HI-CRISPR/Cas9-based technology is the legislation put in place to regulate the development and commercialization of genetically modified organisms (GMOs) in the EU, and which handles issues of uncertainty and safety (Sprink et al. 2016). The Directive 2001/18/EC defines a GMO as an organism ''in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'', and GM techniques are further described as ''recombinant nucleic acid techniques involving the formation of new combinations of genetic material''. In our opinion, haploid production through CRISPR/Cas9 mutants used for HI needs to be exempt from this regulation, because the resultant GMO-free haploid line would never have recombinant DNA incorporated into its single progenitor cell.

The US regulatory framework would categorize HI-CRISPR/Cas9 as a null segregants, because transgenic sequences are eliminated from the final product. However, this technique has the precedent of centromere-mediated chromosome elimination (CCE) through a *GFP-tailswap* transgenic line for which the Plant Health Inspection Service (APHIS) determined that the progeny obtained from CCE would not be subjected to its regulation (Camacho et al. 2014).

Nevertheless, as pointed out by Abbott (2015) and Camacho et al. (2014), the EU and the US criterion in the current legislation are based on the process rather than the product. Therefore, a process that uses recombinant technology could create controversy for regulatory institutions, despite the impossibility of detecting the haploid origin. The regulatory framework should be based on science and able to evolve together with the scientific knowledge and technologies.

Ploidy-level identification of plant material

Ploidy identification of the plants produced during the DH process is essential before the chromosome-doubling treatment to determine whether the plant material has undergone spontaneous duplication, and afterwards to check whether or not the antimitotic treatment has successfully doubled the plants' chromosomes. There are multiple methods to check ploidy level: cytologically, morphologically, via marker-assisted selection or via flow cytometry method.

Cytological procedures for ploidy level determination can be carried out by counting chromosomes or examining the epidermal tissue of the leaves. Chromosome counting usually uses root tip cells, which are fixed, and the chromosomes are then stained and observed for counting (Maluszynska 2003). Cytological analysis of leaves correlates chloroplast number, stomata dimensions, and size with ploidy level. However, applying chromosome-counting methods to species with small chromosome size is very time consuming and difficult, and chloroplast and stomata analysis is species- and genotype-dependent. Despite this, results from both cytological procedures are extremely accurate and sample preparation and staining is easy and fast.

Haploid and diploid plants can also be distinguished according to morphological observations of the plant material. Morphological observation is based on comparing plant traits of the donor plants and regenerants, such as: height, vigor, leaf shape, flower development, fertility, and presence of pollen. This methodology does not require special equipment, but it is sometimes unreliable and subject to environmental effects. In the *Cucurbitaceae* family, leaf morphology, flower shape and size, pollen production, stem length or node number, are phenotypic variations that can be analyzed for ploidy determination (Dong et al. 2016). Couto et al. (2013) correlated haploid levels with small plant size and brittle leaves, but excluded ploidy determination of haploid seeds via morphometric parameters due to the great variability in the seeds. Yuan et al. (2015) detected haploid individuals in two *Brassica* species due to the weak growth of the haploids and the small size of the plants as wells as the presence of smaller flower buds, the absence of pollen, and a lack of stamens in the flowers.

The use of segregating alleles in the donor parent is another methodology for ploidy level determination via marker-assisted selection. Simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP) marker analysis techniques are ideal to identify ploidy level as well as homozygosity in spontaneous DH regenerants

or heterozygosity in diploid somatic regenerants. For instance, in soybean, 114 androgenic embryo-like structures were analyzed for the Satt418 SSR marker, and 74% were found to be heterozygous originating from somatic anther tissue (Rodrigues et al. 2004). In pumpkin, 23 SSR markers were screened in 253 parthenogenetic diploid plants and the results showed no spontaneous chromosome doubling (Košmrlj et al. 2013). In oat, (Kiviharju et al. 2017) DNA markers were used for selection and this indicated that 3.4% of regenerated androgenic plants were heterozygous.

Despite all the methods described above for ploidy-level determination, flow cytometry is the most used method for a wide range of species because of its convenience and rapidity estimating the nuclear genome size in plants (Doležel et al. 1998). Estimation of ploidy-level via nuclear suspensions of plant cells and chromosome staining has been used in DH programs for routine laboratory analysis. Many optimizations have been done to increase the number of nuclei in suspension using extraction methods such as chopping and bead beating, alongside with numerous modifications of isolation buffers and staining procedures (Doležel and Barto 2005; Hooghvorst et al. 2019a, b). Moreover, in addition to haploid and diploid cells, flow cytometry is especially useful for detecting triploid, tetraploid, and mixoploid plants. On the other hand, flow cytometry requires highly specialized equipment that is not required with earlier methods of ploidy determination.

Conclusions

The attempt of plant breeding and DHs to increase productivity and other important plant traits makes it necessary to continue refining existing and new methods to finally achieve a more sustainable production according to social needs. We have undertaken an analysis of DH production methods coupled with chromosome-doubling protocols for production of DH plants in major crops. We have drawn general conclusions about the success of different approaches to DH generation and the implications of the existing technologies of DH production and chromosome doubling for future research. DH technology has been a major boost for plant breeding, reducing the time and labor required to derive new breeding varieties. Among the DH processes, chromosome doubling is often overlooked due to the importance of the haploid induction step or the high frequency of spontaneous DHs, and this is reflected in the absence of DH protocols or a lack of efficiency in those that exist. Androgenesis via anther culture is the most common protocol for haploid and DH plant production, being the predominant method in vegetable and horticultural crops, and the only method used in cereals. The chromosome-doubling step is far from being settled and there is a need to continue investigating new protocols based on new or existing antimitotic compounds to reduce toxicity-related mortality and to attain higher frequencies of chromosome doubling. Induced chromosome doubling has a genotypic dependency, and even species with a high rate of spontaneous doubling should not be ignored when developing efficient chromosome-doubling methods because some genotypes are unable to regenerate spontaneously. Inducing haploids using genome editing via the CRISPR/Cas9 system could revolutionize the whole process of haploid generation and DH production, and should have an impact on plant breeding in the coming years to parallel the early days of the DH technique. For crops with a short progress on DH technology, CRISPR/Cas9 HI approach could open a new insight, allowing the production of pure homozygous lines. For many of the vegetable crops and secondary cereals, like tomato, Fabaceae, Cucurbitaceae, barley, oats, rye and triticale, HI systems will allow researchers to avoid the need for wide ranging adaptation of protocols to different genotypes, which is a highly time-consuming pathway requiring much trial and error. The only adaption required to the tissue culture technique will be the regeneration of CRISPR/Cas9 genome-edited plants, which will be a much easier task because only one genotype will be sufficient as the pollen donor for haploid induction across a diversity of receptor genotypes of the same species. In the future, HI-mediated genome-editing CRISPR/Cas9 system should be exempted from GMO legislation due to the absence of GMO in the maternal antecessor and haploid descendancy. The uprising CRISPR/Cas9-based gene targeting approach for haploid induction will make the chromosome-doubling step inexorable, because of the low or absent spontaneous chromosome doubling in haploid induction process. Therefore, new in vitro or in vivo chromosome-doubling protocols will be needed for species where haploid induction has not yet been reported due to low regeneration efficiency or a complete lack of regeneration success.

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Compliance with ethical standards

Conflict of interest IH and SN declare no conflict of interest.

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CHAPTER 4. Opportunities and Challenges in Doubled Haploids and Haploid Inducer-Mediated Genome-Editing Systems in Cucurbits

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Opportunities and Challenges in Doubled Haploids and Haploid Inducer-Mediated Genome-Editing Systems in Cucurbits

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Abstract: Doubled haploids have played a major role in cucurbit breeding for the past four decades. In situ parthenogenesis via irradiated pollen is the preferred technique to obtain haploid plantlets whose chromosomes are then doubled in Cucurbitaceae, such as melon, cucumber, pumpkin, squash and winter squash. In contrast to doubled haploid procedures in other species, in situ parthenogenesis in cucurbits presents many limiting factors which impede efficient production of haploids. In addition, it is very time-consuming and labor-intensive. However, the haploid inducer-mediated genome-editing system is a breakthrough technology for producing doubled haploids. Several reports have described using the CRISPR/Cas9 system in cucurbit species, and although its application has many bottlenecks, the targeted knock-out of the CENH3 gene will allow breeders to obtain haploid inducer lines that can be used to obtain parthenogenetic embryos. In this review, we discuss the progress made towards the development of doubled haploids and haploid inducer genotypes using CRISPR/Cas9 technologies in cucurbit species. The present review provides insights for the application of haploid inducer-mediated genome-editing system in cucurbit species

Keywords: cucurbits; doubled haploids; parthenogenesis; genome editing; CRISPR/Cas9; haploid-inducer

1. Introduction

The Cucurbitaceae is a family with several important crop species and contains a great genetic, morphologic and phenotypic variability. In terms of worldwide production, the most important species are watermelon with 104 m tons, cucumber and gherkins with 75 m tons, pumpkin, squash and gourds with 28 m tons and melon with 24 m tons [1]. Commercial cultivars are usually F1 hybrids due to heterosis, which results in earlier harvest, increased yield and higher vigor [2].

Therefore, pure lines used as parents for hybrid production are invaluable. There are two main methods in cucurbits to produce completely homozygous lines, classical breeding and doubled haploids. Classic breeding requires several rounds of selfing and selection for eight to ten generations, which consumes a considerable amount of time and resources. On the other hand, doubled haploid (DH) methodology allows the efficient production of completely homozygous pure lines in less than two years. Parthenogenic DH methodology in cucurbits consists of two basic steps, the initial production of haploid material through in situ parthenogenesis via pollination with irradiated pollen and the subsequent chromosome doubling of haploid plants to restore the diploid chromosome content and to allow the generation of DH seed. Nevertheless, parthenogenesis in cucurbits has

several bottlenecks and limitations that may jeopardize the final production of DH lines, such as high genotypic dependency; low fruit set when pollinated with irradiated pollen; difficulties in detecting parthenogenetic embryos; low production of parthenogenetic embryos; recalcitrant culture in vitro; high mortality of parthenogenetic plants during in vitro culture and after the chromosome doubling treatment; low rates of chromosome doubling; and low fruit set of DH lines once chromosomes are doubled [3].

DHs have been widely used to obtain stabilized homozygous lines to be used as parental lines to produce F1 hybrids. On the other hand, stabilized lines have been used for: establishing chromosome maps and whole genome; bulked segregant analysis (BSA); and, for mapping quantitative trait loci (QTLs) [4].

Until recently, classical breeding and DHs were the main pathways for the production of parental lines. However, the haploid inducer-mediated genome-editing system is a breakthrough approach that could lead to a new era of cucurbit breeding. The currently-predominant genome editing technique is CRISPR/Cas9 due to its efficiency, versatility, and unprecedented control over mutation. CRISPR/Cas9 consists of a Cas9 nuclease guided by a 20-nt sequence (gRNA). This system induces DNA double-strand breaks (DSBs) that are repaired by either non-homologous end-joining (NHEJ) or homology directed repair (HDR), generating insertion and deletion events (INDELs) [5].

There are numerous reports and reviews of DH production and CRISPR/Cas9 applications in many plant species including cucurbits. Nevertheless, haploid inducer-mediated genome-editing systems have not yet been applied in cucurbits. Therefore, the purpose of this review is to focus on haploid inducer-mediated genome-editing systems in cucurbit species to provide new insights, opportunities and challenges, which may be valuable for developing this technique in cucurbits and other species.

2. Doubled Haploids Procedure in Cucurbits

Doubled haploid lines in cucurbit species can be produced by parthenogenesis, androgenesis or gynogenesis. However, parthenogenesis is the currently-predominant technique to produce doubled haploids in cucumber, melon, watermelon and pumpkins [6]. The parthenogenesis process starts with in vivo pollination using irradiated pollen. The resulting parthenogenetic embryos are then detected, rescued and cultured in vitro, in order to germinate and develop into plantlets. The ploidy-level of parthenogenetic plantlets is estimated by flow cytometry and can result in haploid, spontaneous doubled haploid, mixoploid or tetraploid levels. The haploid and mixoploid plantlets need to undergo in vitro or in vivo chromosome doubling, usually using colchicine as antimitotic compound. Once the plantlets have doubled their chromosomes, they are cultured in the greenhouse together with the spontaneously-doubled haploid lines to recover DH seed [3,6,7]. During the DH process, a high genotypic dependency and other factors continuously hamper each step causing a loss of efficiency that might be critical.

2.1. Pollination with Irradiated Pollen and Fruit Set

The first step of the in situ parthenogenetic process is the irradiation of mature pollen and the pollination of the receptor female flowers. γ-ray (^{60}Co or ^{137}Cs) and soft X-ray are the usual irradiation sources applied to male flowers. The ionized pollen can germinate on the female stigma and grow pollen tubes to reach the embryo sac. However, this pollen is genetically inactive and unable to fertilize the egg-cell and the polar nuclei. Therefore, irradiated pollen stimulates egg-cell division and parthenogenetic embryo induction [3]. Overall, the dose of ionizing radiation can range from 25 to 500 Gy, depending on the species and can yield less parthenogenetic haploid embryos at higher or more diploid embryos at lower dosages. Therefore, irradiation should be optimized for each species because pollen sensitivity is attributed to radiation-resistance.

The parameters that define the success or failure of pollination with irradiated pollen are the number of fruits set and the ratio of parthenogenetic embryos per fruit. Fruit set is lower when

pollination is performed using irradiated pollen [3]. The number of female flowers that develop into a fruit can range between 10–25% in melon [3], 20–25% in pumpkin [8] or 50% in cucumber [9]. The initial number of parthenogenetic embryos is crucial to have enough plant material during the whole process. The plant material usually decreases progressively during each step of the DH process, due to mortality, inefficiency of the method and recalcitrant performance. Frequently, the ratio of parthenogenetic embryos per fruit is low, 0.23–5.79 in cucumber [9,10]; 0.2–16 in pumpkin, squash and winter squash [8,11–14]; 0.3–6 in melon [3,6]; or 1.4 in watermelon [15].

In addition, the genotype of the donor and the receptor plants have an influence in the fruit set. For instance, inbred lines of cucumber resulted in a higher number of parthenogenetic embryos than hybrid lines [16]. On the other hand, the growing environment is another key element to take into account when pollinating with irradiated pollen. During summer/spring the fruit set and the number of embryos is usually higher than in winter/autumn [3,17].

2.2. Embryo Detection and Rescue

The use of irradiated pollen to pollinate allows the production of fruits potentially containing parthenogenetic embryos in some of their seeds. However, the vast majority of seeds are empty [3]. Therefore, before embryo rescue, embryo have to be detected to be excised from the seed and cultured in vitro. Three different methods can be used to detect and rescue the parthenogenetic embryos: inspection of seeds one-by-one, X-ray photography and culture of seeds in liquid medium. Each one differs in the time invested, the efficiency and the required equipment (Table 1). The inspection of seeds one-by-one with the help of a binocular microscope is the most widely-used method because it does not require specialized equipment and successfully detects parthenogenetic embryos. Moreover, a light box can be used to ease the inspection of seeds [3,18]. Nevertheless, the inspection of seeds one-by-one is very laborious and time-consuming. On the other hand, the X-ray radiography is the most straightforward method due to is much faster than the inspection of seeds, but requires specialized equipment which is not always available in all laboratories [10]. Lastly, the culture of seeds in liquid medium has been frequently shown to fail, due to contamination with endophytic bacteria and fungi [3,5].

Table 1. Summary of the parthenogenetic methodology and efficiency in cucurbit species for doubled haploid (DH) line production.

Species	Embryo Detection Method	Embryos per Fruit	Mortality Rate In Vitro	Ploidy-Level	Chromosome Doubling		Reference
					Method	Efficiency	
Cucumber	X-ray	0.23	68.23%	62% H 38% M	E20H8 medium suppl. 500 µM colchicine for 48 h	55% M 30% DH	Claveria et al. (2005) [10]
Cucumber	One-by-one	5.79	79.73%	-	-	-	Smiech et al. (2008) [9]
Melon	One-by-one	6.27	30.85%	73% H 27% M	In vitro solution 500 mg·L^{-1} colchicine for 3 h	26% DH	Lim and Earle (2008) [7]
Melon	X-ray	0.30	50.94%	73.1 H 23.1% DH 3.8% M	In vivo solution 5000 mg·L^{-1} colchicine for 2 h	9.38% M 20.31% DH	Hooghvorst et al. (2020) [3]
Melon	One-by-one	1.97	34.22%	90% H	E20H8 medium suppl. 500 µM colchicine for 48 h	-	Gonzalo et al. (2011) [19]
Melon	-	-	-	-	In vivo solution 0.5% colchicine for 2 h	46.03% DH	Solmaz et al. (2011) [20]
Pumpkin	One-by-one	16.38	84.04%	5.9% H	-	-	Kurtar et al. (2009) [13]
Pumpkin	One-by-one	69.85	-	0.86% H			Košmrlj et al. (2013) [8]
Squash	One-by-one	0.2–10.5	71.2–80.1%	43.7% H 56.3% D	-	-	Kurtar et al. (2002) [12]
Squash	One-by-one	18.45	-	65.6% H			Baktemur et al. (2014) [14]
Watermelon	One-by-one	1.40	-	-	-	-	Taskin et al. (2013) [15]
Winter squash	One-by-one	13.72	85%	10.9% H	-	-	Kurtar and Balkaya (2010) [11]

H haploid, *M* mixoploid, and *DH* doubled haploid.

2.3. In Vitro Culture

The detected parthenogenetic embryos are rescued and cultured in vitro in specific media. Several media can be used to culture embryos in vitro successfully such as E20A medium [21], MS [22], N6 [23] and CP [24]. Nevertheless, E20A medium with or without modifications is the most commonly used medium for parthenogenetic embryo culture. The parthenogenetic haploid embryos have shorter and irregular cotyledons in comparison to diploid embryos. In addition, they can present a range of morphogenic shapes and stages (pointed, globular, arrow-tip, torpedo, heart, cotyledon, amorphous or necrotic). The survival, germination and development of parthenogenetic embryos is usually correlated with the shape and if its white or necrotic [11]. In addition, during the in vitro process there is a high selection pressure because of deleterious gene combination in homozygosis that can be responsible of vegetative growth problems and can hamper the germination and plantlet development [6,7]. Then, the germination of embryos and the growth and development of the parthenogenetic plantlets is not always guaranteed. The mortality rate of embryos and plantlets in vitro is dramatically high, 30–85% (Table 1).

2.4. Chromosome Doubling

Plantlets that survive parthenogenesis are usually haploid (~70%), but mixoploid or even spontaneous DHs can be obtained too [3,5]. Spontaneous DH plantlets do not need to undergo chromosome doubling and can be directly acclimatized for DH seed recovery. On the other hand, haploid and usually mixoploid plantlets have to be chromosome doubled with antimitotic compounds. In cucurbit species, chromosome doubling has been reported chiefly in melon and cucumber. The doubling treatment can be in vitro or in vivo. The antimitotic compound can be colchicine, oryzalin or trifluralin. However, it is mostly performed using colchicine. Dinitroanilines, oryzalin and trifluralin, have been reported as successful and very promising in cucumber [25] and unsuccessful in melon 'Piel de Sapo' [3]. A high mortality might be recorded after the treatment, due to the toxicity of colchicine or the hyperhidricity suffered by the explants. On the other hand, in vivo treatment applies higher concentration of colchicine for a shorter time (2–3 h) by immersing the apical meristems of plants growing in the greenhouse into the colchicine solution. Chromosome doubling is highly influenced by the genotype and the most suitable method must be determined empirically. In melon, the efficiency of chromosome doubling in "Piel de Sapo" type genotype was higher when applying colchicine at 5000 mg·L^{-1} for 2 h in vivo rather than at 500 mg·L^{-1} for 12 h in vitro [3], or in a BC4F1 population, the 26% of haploids chromosome doubled when exposing shoot tip explants to 500 mg·L^{-1} for 3 h in vitro [7]; in cucumber, colchicine was applied in vitro in solid E20H8 medium for 48 h or by submerging plantlet nodes and tips into a colchicine solution for 3–12 h reaching a 55% of chromosome doubling [10] (Table 1).

2.5. DH Seed Recovery

Pure and viable seed must be recovered from chromosome doubled and the spontaneous DHs plants. Those plants present a low fruit set (~3–10%) when are self-crossed due to a low germination ability of pollen and the presence of different ploidy-levels in the whole plant [26]. Abnormal ploidy-level in the same plant can be observed in the female and male flowers during the process. The self-crossing must be by hand-pollination to avoid external pollinations. In addition, the germination of DH seed is difficult and a generation for seed multiplication is usually required prior to F1 hybrid seed production.

3. Genome Editing in Cucurbit Species

Genome editing techniques have the ability to introduce mutations in the plant genome. There are three main site-specific genome-editing nucleases with the capacity to target precise regions of the genome: zinc finger nucleases (ZFN), transcription activator-like effector nucleases

(TALENs) and clustered regularly interspaced short palindromic repeats associated to nuclease Cas9 (CRISPR/Cas9). The CRISPR/Cas9 system has risen as the preeminent genome editing technique, due to its versatility, efficiency and ease to engineering in comparison to ZFN and TALENs. In cucurbits, there are no reports describing the application of ZNF nor TALENs, as far as the authors know. Nevertheless, CRISPR/Cas9 has been applied successfully in cucumber [27,28], watermelon [29–31] and melon [32].

For the success of a CRISPR/Cas9 experiment, breeders need the sequenced genome of the target specie available, an adequate *Agrobacterium*-mediated transformation protocol and an efficient binary vector containing the sequence of the Cas9 protein capable to induce target mutations. In cucurbit species, the genomic sequences of cucumber, watermelon, melon, pumpkin, zucchini, squash, winter squash, bottle gourd and others are available [33]. Several CRISPR/Cas9 binary vectors have been reported successful in cucumber, watermelon and melon, and may be used for other cucurbits (Table 1). However, *Agrobacterium*-transformation protocol is still the main bottleneck to apply genome editing in cucurbits [31,32].

The production of genome-edited plants in cucurbit species usually starts with the selection of the gRNAs targeting the gene of interest and the construction of the CRISPR/Cas9 binary vector. The CRISPR/Cas9 vector containing the gRNAs should be tested in protoplast prior transformation in order to corroborate the gene editing. The verification of the mutation induction before the *Agrobacterium*-mediated transformation process may ensure its success.

3.1. Agrobacterium-Mediated Transformation

The regeneration of transgenic plants in cucurbit species is considered a very recalcitrant process [29,32,34]. To successfully obtain of transgenic plants, regeneration and transformation have to take place. In cucurbits, cotyledonary explants are the main source of plant material for direct organogenesis, which is the production of adventitious buds or shoots from explants without a callus phase. Organogenesis is usually high in cucurbit species when using a suitable regenerating medium. Nevertheless, transformation, the acquisition of the transgene by the germinative cells, is highly inefficient. Therefore, during the process of *Agrobacterium*-mediated transformation, many plants regenerate but the vast majority lacks the transgene. The non-transformed regenerants (so-called "escapes") frequently grow into selective medium, contrary to what occurs in other species. The percentage of "escapes" can be ~30% and are a hindrance in order to select positive transformants. Moreover, the transformation process is usually optimized for and restricted to a few genotypes. This impedes the application of transformation and genome editing to a wide range of genotypes of interest. In comparison to the transformation efficiency of other species such as rice, *Arabidopsis* or tomato, cucurbit species have a low transformation of 1.32–5.63% (Table 2).

Table 2. Summary of the methodology and efficiencies of CRISPR/Cas9 reported experiments in cucurbit species.

Species	Target Gene	Plant Material	Transformation		Genome Editing Efficiency	Reference
			Method	Efficiency		
Cucumber	eIF4E	Cotyledonary explants	*Agrobacterium*-mediated	-	20%	Chandrasekaran et al. (2016) [28]
Cucumber	WIP1	Cotyledonary explants	*Agrobacterium*-mediated	1.32%	65.2%	Hu et al. (2017) [27]
Watermelon	PDS	Cotyledonary explants	*Agrobacterium*-mediated	1.67%	100%	Tian et al. (2017) [29]
Watermelon	PDS	Protoplast	Protoplast transfection	-	42.1–51.6%	Tian et al. (2017) [29]
Watermelon	ACS	Cotyledonary explants	*Agrobacterium*-mediated	-	23%	Tian et al. (2018) [30]
Watermelon	PSK1	Cotyledonary explants		2.3%	-	Zhang et al. (2020) [31]
Melon	PDS	Cotyledonary explants	*Agrobacterium*-mediated	5.63%	42–45%	Hooghvorst et al. (2019) [32]
Melon	PDS	Protoplast	Protoplast transfection	-	25%	Hooghvorst et al. (2019) [32]

In addition, the ploidy-level of T0 generation can be spontaneously duplicated during the direct organogenesis resulting in tetraploid regenerants. Those polyploid plants are sterile and selfing is impossible for segregation of the transgene or for transgenic seed multiplication.

3.2. Genome Editing Efficiency

The mutation efficiency depends on the GC content of the sgRNA, the number of transformed cells and the Cas9 protein expression level in transgenic cells [35]. In species such as rice or Arabidopsis, the efficiency frequently ranges between 80–100% and in cucurbit species it ranges from 20–100% (Table 2). Taking into account the larger effort invested in rice or Arabidopsis to apply and optimize CRISPR/Cas9 in comparison to cucurbit species, CRISPR/Cas9 efficiency in cucurbits can be recognized as acceptable and suitable. Then, genome editing efficiency is not a bottleneck when applying CRISPR/Cas9 in cucurbits. Nevertheless, further attempts should be assayed, as done in other species, to achieve a higher genome editing efficiency such as using endogenous promoters of Cas9 and sgRNA expression, heat treatment during transformation, optimization of transformation efficiency [36,37].

4. Haploid Inducer-Mediated Genome-Editing in Cucurbit Species

Haploid inducer approach is based on an intraspecific cross between a haploid inducer line and a receptor genotype of interest from where to obtain haploid lines. The haploid inducer line carries a specific mutation in an essential gene for the normal fertilization of female cells and therefore induces the parthenogenetic development of haploid embryo from the egg cell. The MATRILINEAL (MATL) gene, also known as NOT LIKE DAD (NLD) or PHOSPHOLIPASE A1 (PLA1) encodes a pollen-specific phospholipase and is usually the mutated gene that causes the haploid inducer ability in cereals [38–40]. For instance, in maize, natural haploid inducer lines were discovered to carry a 4-bp insertion in the carboxy terminus of the MATL gene. Natural haploid inducer lines have been used in wheat, maize, tobacco or barley for years to induce a parthenogenetic process for further haploid line obtention [40–44]. In cucurbit species, no natural haploid inducer lines have been described, as far as the authors know. However, with the recent genome editing tools available, breeders have the possibility to generate haploid inducer lines through genome-editing techniques. Haploid inducer lines have been produced in cereal species mutating MATL gene via CRISPR/Cas9. Haploid inducer-mediated genome-editing approach yielded a 6.7% of parthenogenetic haploids in maize [45] and ~6% in rice [46]. Those rates represent a great improvement in in vivo doubled haploid obtainment in those species to allow the acceleration of breeding.

The MATL gene is present and highly conserved in cereal species but not in dicots. Therefore, the haploid inducer-mediated genome-editing system in dicots usually targets centromeric histone 3 (CENH3) gene. The CENH3 gene codes a histone present in all plants that determines the position of the centromere, and, thus, plays a major role in chromosome segregation during mitosis [47]. The haploid inducer related potential of CENH3 gene was first discovered in *Arabidopsis*. In this study, a haploid inducer line named "green fluorescent protein (GFP)-tailswap" carried GFP fused to the N-terminal tail domain of an H3 variant, replacing the N-terminal tail of CENH3. This CENH3 mutant line produced a haploid induction rate of 25–45% when crossed with a CENH3 wild type line by chromosomes elimination of the mutant line (Table 3) [48]. Since the evidence of the potential of CENH3 for haploid induction, some applications have raised using ethyl methane sulfonate (EMS) mutagenesis targeting CENH3 gene to obtain haploid inducing lines, such as in tomato with a haploid progeny of 0.2–2.3% (WO 2017 200386/KEYGENE); in cucumber, with an efficiency of 1%; and in melon, with an efficiency 1.5% (WO 2017 081,011 A1/RIJK ZWAAN) (Table 3). However, those procedures are patented, and EMS mutagenesis is quite inefficient in those species.

Therefore, the generation of haploid inducer lines in cucurbit species may be democratized targeting CENH3 using CRISPR/Cas9, due to the specificity of CRISPR/Cas9 in multiple sequences of the CENH3 gene which can be targeted efficiently. However, for the generation of haploid inducer lines mutated via CRISPR/Cas9 a successful transformation protocol would be imperative at least for one genotype. Generally, transformation protocol is adjusted for a specific genotype in cucurbit species. Fortunately, the genotype of the haploid inducer line is not an issue for haploid induction as long as it is the same species as the donor genotype from where to recover haploid embryos and bears male flowers. From the T0 CRISPR/Cas9 generation, heterozygous CENH3-mutated should be self-crossed in order

to select: (i) T1 transgene-free and homozygous CENH3-mutated individuals, and (ii) transgene-free and heterozygous mutated-CENH3 T1 individuals. The homozygous CENH3-mutated lines will be used for haploid induction process and heterozygous lines for haploid inducer line maintenance for future applications. Through this method, haploid inducer lines may be successfully maintained and used over the time (Figure 1).

Table 3. Haploid induction reports mediated by mutation or disruption of centromeric histone 3 (CENH3), MATRILINEAL (MATL) or DMP.

Species	Target Gene	Mutation or Disruption Method	Haploid Progeny	Reference
Arabidopsis	CENH3	GFP-tailswap disruption	4–34%	Ravi and Chan (2010) [48]
Arabidopsis	CENH3	GFP-tailswap disruption	2.50%	Kuppu et al. (2015) [49]
Arabidopsis	CENH3	GFP-tailswap disruption	4.80%	Karimi-Ashtiyani et al. (2015) [50]
Arabidopsis	CENH3	GFP-tailswap disruption	3.60%	Kelliher et al. (2016) [51]
Arabidopsis	DMP	CRISPR/Cas9	2.1%	Zhong et al. (2020) [52]
Cucumber	CENH3	EMS-induced mutation	1%	WO 2017 081,011 A1/RIJK ZWAAN
Maize	CENH3	GFP-tailswap disruption	3.6%	Kelliher et al. (2016) [51]
Maize	MATL	TALEN	6.70%	Kelliher et al. (2017) [45]
Maize	DMP	CRISPR/Cas9	0.1–0.3%	Zhong et al. (2019) [53]
Melon	CENH3	EMS-induced mutation	1.50%	WO 2017 081,011 A1/RIJK ZWAAN
Rice	CENH3	EMS-induced mutation	1%	WO 2017 200386/KEYGENE
Rice	MATL	CRISPR/Cas9	2–6%	Yao et al. (2018) [46]
Tomato	CENH3	GFP-tailswap disruption	0.2–2.3%	WO 2017 200386/KEYGENE
Wheat	MATL	CRISPR/Cas9	18.9%	Liu et al. (2019) [54]

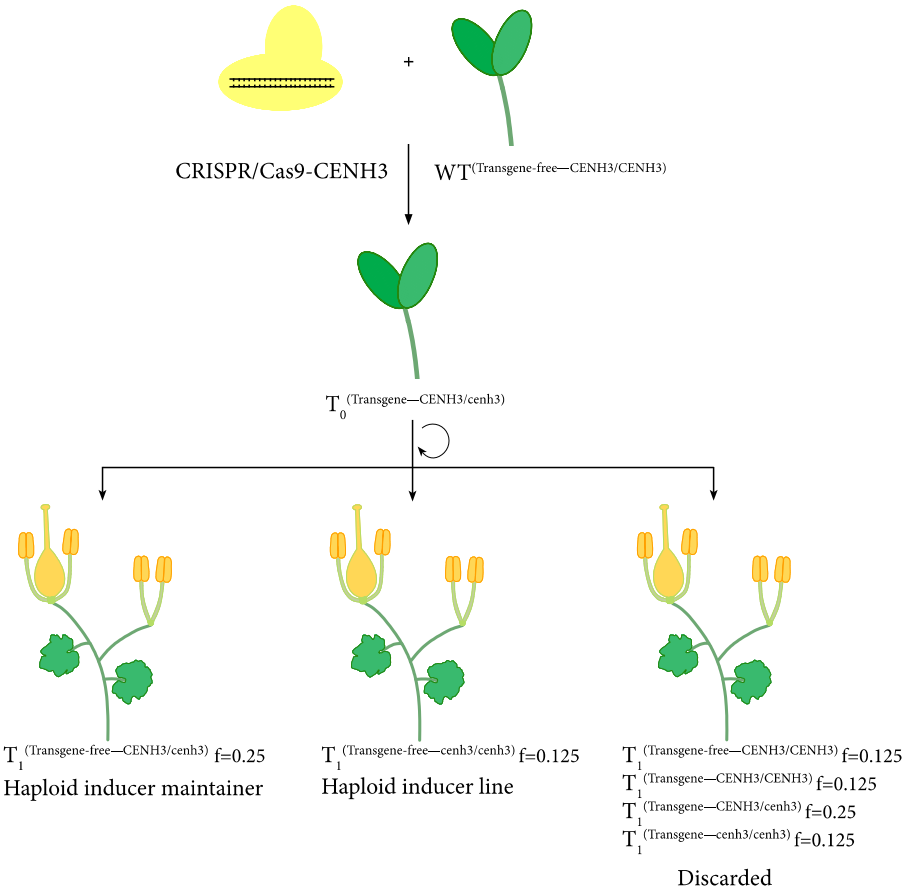

Figure 1. Schematic representation of the obtention of a haploid inducer line and its maintainer. WT: wild type.

The application of the haploid inducer-mediated genome-editing system in cucurbit species could reduce the actual time-consuming, labor-intensive and limited parthenogenic process. Although the process would remain very similar to parthenogenetic process with irradiated pollen due to the initial in vivo pollination with pollen of the haploid inducer line, line, the rate of haploid embryo induction could be significantly improved since no aggressive treatment, such as ionization, would be applied in haploid inducer line pollen. Furthermore, no irradiation source requiring equipment not always available in all laboratories will be needed. On the other hand, parthenogenesis via irradiated pollen is highly genotype-dependent and accomplishment of irradiation depends upon the radiation resistance of the pollen. In addition, some genotypes are reported to be very recalcitrant to induction of haploid generations when pollinated with irradiated pollen. Pollination with pollen of the haploid inducer lines could broaden the range of genotypes that can produce haploids and increase the number of parthenogenetic embryos. This must be assayed to see whether haploid inducer lines can increase the number of haploid embryos produced in cucurbits.

Moreover, parthenogenesis with irradiated pollen is routinely applied in cucumber and melon and substantial progress has been made. In contrast, less effort has been applied in species such as watermelon, winter squash, pumpkin or bottle gourd and, therefore, less progress has been made in optimizing their parthenogenetic protocols. In cucurbit species, the fruits set once pollinated with irradiated pollen or pollen from haploid inducer lines will follow the same steps as in in situ parthenogenesis via irradiated pollen. Consequently, limiting factors described for parthenogenesis with irradiated pollen would likewise be present using the haploid inducer approach. Therefore, the haploid inducer-mediated genome-editing approach can take advantage of the progress made to successfully obtain doubled haploid lines.

The haploid inducer-mediated genome-editing approach is an opportunity to improve the efficiency of doubled haploid production in recalcitrant species. The use of matl or cenh3 mutant lines in cereals or dicots for haploid induction and production might avoid androgenesis, gynogenesis or parthenogenesis via pollen irradiated and boost their obtention in several species.

5. Regulatory Landscape for the New Generation of Doubled Haploids

The development of DH lines has never been restricted by regulatory limitation, because they are obtained using in vitro protocols for haploid generation or in vivo pollination with pollen mutagenized with gamma rays. On the other hand, since the Directive 2001/18/EC put in place in EU, GMOs have been strongly limited because of uncertainty and safety. The GMO definition is "as an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination". The European highest court handed a setback regulation on 25 July of 2018, subjecting organisms obtained using CRISPR/Cas9 and other genome editing techniques under the same regulation as GMOs, defining them as "recombinant nucleic acid techniques involving the formation of new combinations of genetic material". Therefore, GMOs and CRISPR/Cas9-derived plants have the same regulation landscape despite the fact that the first added a transgene essential for the trait improvement whereas the second used recombinant DNA for trait improvement and subsequently lost it through segregation. Then, as pointed out by Abbot, the EU criterion is based on the process and the product when using recombinant DNA [55].

Doubled haploids derived from haploid inducer-mediated genome-editing approach presents a challenge in terms of regulation. When haploid lines are produced by crossing the haploid inducer line with a receptor genotype, the chromosomes of the haploid inducer line are eliminated, and the haploid-derived progeny carries the maternal set of chromosomes. The only progenitor of the haploid generation has never carried recombinant DNA and has no improved traits derived from mutations induced by the genome editing technique. The origins of haploid lines derived from haploid inducer or irradiated pollen are impossible to trace. Therefore, haploid-mediated genome-editing system should not be restricted by any limitation or regulation.

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ANNEX II – OTHER PUBLISHED ARTICLES

Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice

Plant Cell, Tissue and Organ Culture (PCTOC)
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ORIGINAL ARTICLE

Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice

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Abstract
Rice double haploid (DH) plants are produced mainly through anther culture. In order to improve the anther culture protocol, microspores of two japonica rice genotypes (NRVC980385 and H28) were subjected to three growth regulator combinations and four colchicine treatments on induction medium. In addition, a post anther culture procedure using colchicine or oryzalin was tested to induce double haploid plantlets from haploid plantlets. A cold pre-treatment of microspores for 9 days at 10 °C increased callus induction 50-fold in the NRCV980385 genotype. For both genotypes, 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin on colchicine-free induction medium gave the best culture responses. The culturability of both genotypes changed on colchicine-supplemented induction media. A high genotype dependency was recorded for callus induction, callus regenerating green plantlets and regeneration of green double haploid plantlets. Colchicine at 300 mg L^{-1} for 48 h enhanced callus induction 100-fold in H28. Colchicine-supplemented media clearly improved green double haploid plantlet regeneration. We showed that the post-anther culture treatment of haploid plantlets at 500 mg L^{-1} of colchicine permitted fertile double haploid plantlets to be generated. Finally, an enhanced medium-throughput flow cytometry protocol for rice was tested to analyse all the plantlets from anther and post anther culture.

Keywords Mediterranean japonica rice · Anther culture · Hormones · Colchicine · Antimitotics · Double haploid

Introduction

Doubled haploid lines (DHs) are produced when spontaneous or induced chromosome duplication of haploid cells occurs. DH plants are complete homozygous individuals that can be produced within a year through anther or microspore culture. Therefore, the production of homozygous lines from heterozygous parents is feasible and shortens the time required to obtain them (Germanà 2011). Nowadays, anther culture is being used to produce DH plants in more than 250 species, including major cereals such as rice, wheat, maize, barley and also economically important trees, fruit crops and medicinal plants (Maluszynska 2003).

Rice DH plant production is mainly obtained through anther culture. Niizeki and Oono (1968) were the first to produce haploid rice plantlets through anther culture. Rice anther culture is a two-step process with initial callus development and subsequent green plantlet regeneration from embryogenic callus (Mishra and Rao 2016). Since the first report of anther culture, much research has aimed at optimizing the media used at each step in the process to enhance callus induction and callus regeneration (Herath et al. 2010; Pauk et al. 2009). This work has focused on overcoming limiting factors that reduce the efficiency of green DH plantlet production such as high genotypic dependency, low frequency of callus induction and plantlet regeneration, the low percentage of doubled haploids

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produced and the high ratio of albino plantlets (Lentini et al. 1997). The application of stress during the developmental period of pollen grains, osmotic stress applied to cells during culture, the composition of the culture media, and the addition of antimitotic agents, gelling agents or growth regulators amongst many exogenous factors may affect the success of anther culture in rice (Mishra and Rao 2016). Endogenous factors such as the rice variety and genotype also affect anther culture success. Indica rice varieties have a limited response to anther culture due to early necrosis, poor callus proliferation and a high regeneration of albino plantlets (Chen et al. 1991), unlike japonica varieties where green DH plant production is more efficient (He et al. 2006).

Despite the improvements and progress achieved in every step of the anther culture procedure, there is still a need to optimize conditions for higher rates of green DH plant production while reducing the amount of work in each step. Colchicine is an antimitotic compound widely used in microspore culture and has been shown to improve results in terms of green double haploid plant production (Forster et al. 2007) in maize (Obert and Barnabás 2004), barley (Thompson et al. 1991), wheat (Barnabás et al. 1991), rapeseed (Weber et al. 2005), and other species. However, few authors have reported the use of colchicine in rice anther culture. Alemanno and Guiderdoni (1994) were the first to study a routine in vitro colchicine treatment to increase DH plant production in rice. In addition, post anther culture procedures have rarely been used in green haploid plantlets regenerated from anther culture. Finally, such a procedure can be undertaken either in vivo by treating tillers with antimitotic compounds such as colchicine in order to increase the DH recovery from haploid plantlets (Jensen 1974; Zapata-Arias 2003; Chen et al. 2002) or in vitro as explained in this work.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-*D*) and naphthaleneacetic acid (NAA) are the most used hormones for rice callus induction from anthers (Trejo-Tapia et al. 2002).

The aim of this study is to improve the anther culture efficiency in two japonica rice genotypes by assessing some factors that could improve the numbers of green double haploid plants. Thus we tested (i) the effect of different growth regulators (2,4-*D*, NAA and Kinetin) in the anther culture induction medium, (ii) the effect of different colchicine doses in the anther culture induction medium and (iii) a post anther culture procedure to increase plant DH production from haploid plantlets through colchicine and oryzalin in vitro treatments. We showed that colchicine-supplemented media increase green plantlet double haploid production and 500 mg L^{-1} of colchicine in a post-anther culture procedure enabled recovery of green double haploid plantlets from haploid plantlets to be maximized.

Materials and methods

Plant material and growth conditions

The commercial temperate japonica rice variety NRVC980385 and a temperate japonica F2 hybrid called H28, provided by the *Càmara Arrossera del Montsià SCCL* cooperative, were used as plant material. Plants were grown in greenhouse conditions at the *Servei de Camps Experimentals* at the University of Barcelona (Barcelona, Spain) in 4 L plastic containers filled with rice substrate as previously described (Serrat et al. 2014).

Anther culture procedure

Tillers were selected at the booting stage, when the distance from the flag leaf to the auricle of the penultimate leaf was 5–12 cm. The time of collection was from 8:00 to 9:30 as recommended by Chen et al. (1991). Collected tillers were soaked in 70% ethanol for 1 min, rinsed twice with distilled water and were then cold pre-treated for 9 days at 10 °C in polystyrene bags, prior to being surface disinfected again as above. Tillers were dissected to obtain the panicles in a laminar flow cabinet. Panicles were soaked in 70% ethanol for 1 min, rinsed twice and soaked in 10% sodium hypochlorite solution with Tween 20 (30 drops L^{-1}) and 35% HCl (50 drops L^{-1}) for 3 min, and rinsed five times in sterile distilled water. Anthers were obtained from the panicles and plated into 90 mm petri dishes (Sterilin LTD, Cambridge). Basal induction medium consisted of Chu N6 modified as follows: N6 standard salts and vitamins fortified with a combination of growth regulators, 1 g L^{-1} casein enzymatic hydrolysate, 250 mg L^{-1} L-proline, 2 mg L^{-1} 500 mg L^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES), 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite.

The anther culture procedure was carried out in parallel for all the combinations of different growth regulators and their concentrations, colchicine concentrations and colchicine exposure times (Table 1). Regarding growth regulators, three combinations were used: (i) treatment D1, 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; (ii) treatment D2, 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, as used by Serrat et al. (2014) and Chen et al. (2002); and (iii) treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin, as used by Alemanno and Guiderdoni (1994). Colchicine was assayed at 0 (control), 150 and 300 mg L^{-1}, each for the two exposure times of 24 and 48 h. Thus, five colchicine treatments were tested and named as concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Six petri dishes were sown with approximately 100 anthers

Table 1 Conditions for induction media assayed for both genotypes

Growth regulator			Colchicine treatment		Conditions
2,4-*D* (mg L^{-1})	NAA (mg L^{-1})	Kinetin (mg L^{-1})	Concentration (mg L^{-1})	Exposure time (h)	Concentration/time-hormone
1	0	1	0	0	0/0-D1
			150	24	150/24-D1
			150	48	150/48-D1
			300	24	300/24-D1
			300	48	300/48-D1
2	0	1	0	0	0/0-D2
			150	24	150/24-D2
			150	48	150/48-D2
			300	24	300/24-D2
			300	48	300/48-D2
0	2	0.5	0	0	0/0-NA
			150	24	150/24-NA
			150	48	150/48-NA
			300	24	300/24-NA
			300	48	300/48-NA

Three growth regulator treatments were tested: *D1* 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, *D2* 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; and treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin; and three colchicine treatments concentrations were applied, 0 (control condition), 150 and 300 mg L^{-1}

for each condition. After 24 or 48 h, anthers inoculated in colchicine-supplemented media were transferred to exactly the same medium but colchicine-free.

Anthers were kept in the dark at 24 °C and analysed weekly for 8 weeks. Microspore-derived calluses of 1–2 mm diameter that emerged from anthers were transferred to callus regeneration medium as described by Serrat et al. (2014). Anthers that induced callus were removed to ensure a count of one callus per anther, to avoid overestimation of callus induction and to match the number of calluses and induced calli for data analysis. Calluses were transferred to plantlet regeneration medium containing Chu N6 (Chu 1975) standard salts and vitamins fortified with 1 g L^{-1} casein hydrolysate, 250 mg L^{-1} L-proline, 1 mg L^{-1} naphthaleneacetic acid, 2 mg L^{-1} kinetin, 500 mg L^{-1} MES, 30 mg L^{-1} sucrose and 3 g L^{-1} Gelrite. IWAKI 94 mm petri dishes (Asahi Techno Glass Corporation, Amagasaki) were filled with 25 mL of the medium. Calluses were transferred after 28 days onto fresh regeneration medium. Cultures were kept at 25 °C and illuminated with 50–70 µmol m^{-2} s^{-1} fluorescent light under a 16/8 h day/night photoperiod until plantlet formation occurred.

The tiny but fully formed albino and green plantlets (0.5-3 cm length) were transferred into tubes with hormone-free MS (Murashige and Skoog 1962) medium as described by Serrat et al. (2014). Subsequently, clearly sprouting individual plantlets were propagated under conditions as described for regeneration above.

All components of the media were supplied by Duchefa Biochemie BV (The Netherlands). Media were prepared using distilled water and the pH was adjusted to 5.7 by adding 1M KOH (Sigma-Aldrich Co). All components including growth regulators were added before standard autoclave sterilization (121 °C for 20 min).

Ploidy-level determination

The ploidy of green and albino regenerated plantlets was determined by flow cytometry following the procedure of Cousin et al. (2009) with slight modifications. About 5 mg of young leaves were collected and put into ice-cold 2 mL microcentrifuge tubes each with a single steel bead (3 mm diameter). To each tube, 300 µL of cold lysis buffer (0.1 M citric acid and 0.5% Triton X-100 in distilled water) were added. Tubes were cooled at − 20 °C for 10 min. Samples were shaken at 25 Hz for a total of 48 s in a MM 400 tissue lyser (Retsch, Haan, Germany). The aliquot from each tube was filtered through a 22 µm nylon filter (Sefar Maissa, Blacktown, Australia), gently vacuumed and transferred to a flow cytometry sample tube (Beckman Coulter Inc., Pasadena, California, USA). Afterwards, 150 µL of propidium iodide (PI) stain solution [0.25 mM Na_2HPO_4, 10 mL 10× stock (100 mM sodium citrate, 250 mM sodium sulfate) and 9 M PI made up to 100 mL with Milli-Q water] was added to each tube. Tubes were then sealed and kept on ice in the dark for 1 h before flow cytometry (FCM) analysis. The stained nuclei samples were analysed using a Gallios™ Flow Cytometer (Beckman Coulter Inc., Pasadena, California, USA) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centres, University of Barcelona) and a 32-well carrousel. One diploid control (NRVC 980385) sample was included every seven measurements. Samples analysed with a clearly defined peak as the reference ploidy control were classified as DH, whereas those producing half the fluorescence were classified as haploids. Flow cytometry data was analysed using Summit Software v4.3 (Beckman Coulter Inc., Pasadena, California, USA).

Diploidization of haploid green plantlets

Green haploid plantlets regenerated from anther culture were subjected to a post anther culture in vitro treatment with colchicine at 1000, 500 and 250 mg L^{-1}, or oryzalin at 5, 2.5 and 1.25 mg L^{-1}; both in a solution containing 1% DMSO and Tween 20 (4 drops·L^{-1}) in sterilized distilled water. Prior to the antimitotic treatment, plantlet stems and roots were trimmed to 3 cm in length and were incubated in the antimitotic solution for 5 h on a shaker at 120 rpm at 25 °C and maintained under sterile conditions in a laminar

flow cabinet. Thereafter, the plantlets were transferred to hormone-free MS medium as described before and by 3–4 weeks of growth plantlets that had survived and reached 10–15 cm in size were collected to perform flow cytometry analysis as described before.

Statistics

All parameters were divided by the number of anthers sown for each treatment and multiplied by 100 in order to obtain percentages: induced calluses (IC), number of calluses regenerating green plantlets (CRGP), number of calluses regenerating albino plantlets (CRALP), number of calluses regenerating green and albino plantlets (CRGAP), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploid plantlets regenerated (GDHPR). The three concentrations of growth regulators in the colchicine-free media were compared with each other, and each colchicine treatment was also compared individually with the corresponding control medium (0/0) according to the growth regulator hormone concentration (D1, D2 and NA). Growth regulators and colchicine treatments were analysed separately for both genotypes. To determine significant differences between the conditions assayed, a Chi-Square ($P < 0.05$) test for homogeneity was used.

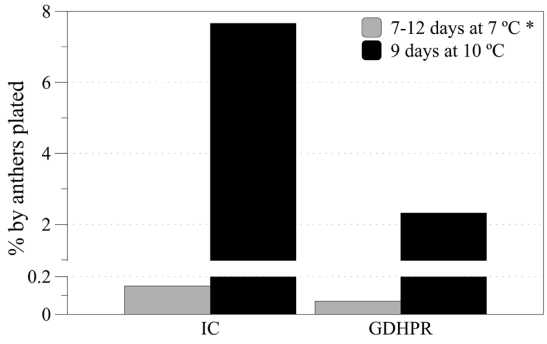

Fig. 1 Induced callus (IC) and green double haploid plantlets regenerated (GDHPR) for NRCV980385 in 0/0-D2 treatment with a cold pre-treatment of 7–12 days at 7 °C* and 9 days at 10 °C (this work). Reproduced with permission from Serrat et al. (2014)

Results

Effect of the cold pre-treatment on callus induction

The percentages of induced calluses (IC) and green double haploid plantlets regenerated (GDHPR) were compared according to the cold pre-treatment applied (Fig. 1) Cold pre-treatment was adjusted to 9 days at 10 °C for NRCV980385 (data not shown) as suggested by Serrat et al. (2014). Following cold pre-treatment for 9 days at 10 °C the IC and GDHPR percentages were 51 times and 33 times higher, respectively, than the 7–12 day pre-treatments at 7 °C.

Effect of different growth regulators on rice anther culture

The culturability of both genotypes with the different growth regulators in colchicine-free induction media is shown in Table 2. In D2 medium, the culturabilities of NRCV980385 and H28 were greater for all the parameters analysed.

For NRCV980385, there were no significant differences ($P > 0.05$) between the three growth regulator treatments (Table 2) for any of the anther culture parameters. Nevertheless, there was a tendency for D2 conditions (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin) to yield higher values than the other treatments for callus induction (IC), callus that regenerates green plantlets (CRGP), regenerated green plantlets (RGP), regenerated double haploid plantlets (RDHP) and regenerated green double haploid plantlets (RGDHP). The values of the D1 and NA growth hormone regulator

Table 2 Culturability results for the temperate japonica rice variety, NRVC980385, and the temperate japonica F2 hybrid, H28, among three growth regulator treatments assayed without colchicine: *D1* 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, *D2* 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; and treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin

Rice genotype	Hormone combination	Anther culture parameters				
		IC	CRGP	GRP	DHPR	GDHPR
NRCV 980385	D1	6.05	0.36	1.96	0.36	1.60
	D2	7.66	0.89	3.21	0.89	2.32
	NA	6.96	0.17	1.39	0.17	1.22
H28	D1	0.18*	0.00	0.00	0.00	0.00
	D2	4.18	0.70	1.57	0.70	0.87
	NA	5.10	0.17	0.51	0.17	0.34

Values followed by * show significant differences at the 5% level in a Chi square test for homogeneity when compared individually with the other two treatments. Induced callus (IC), number of calluses regenerating green plantlets (CRGP), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploids plantlets regenerated (GDHPR)

treatments were similar, but the D2 treatment had slightly higher values for the majority of the parameters.

The percentage of induced calluses (IC) in H28 with the D1 treatment was significantly lower ($P < 0.05$) than the D2 and NA treatments. Therefore, in D1 treatment, parameters that are dependent on IC (CRGP, RGP, RGDHP and RGDHP) were zero, due to a low callus induction. No culture parameters between D2 and NA for H28 were statistically significantly ($P < 0.05$). Although D2 showed higher values than NA for callus that regenerates green plantlets (CRGP), regenerated green plantlets (RGP), regenerated double haploid plantlets (RDHP) and regenerated green double haploid plantlets (RGDHP), these differences were not significant ($P < 0.05$).

Effects of colchicine treatment on callus induction and plantlet regeneration

Callus induction was observed in almost all conditions assayed for both genotypes with the colchicine-supplemented treatments (Table 3). The only exceptions were the 150/24-NA and 300/24-NA media for NRCV980385, with both media used as 24 h colchicine treatments. On one hand, IC in NRCV980385 in the colchicine treatments was significantly lower ($P < 0.05$) than the control. On the other hand, IC for H28 seemed to increase with colchicine, showing significant differences ($P < 0.05$) in both colchicine treatments over 48 h. Moreover, the values were higher in comparison to their respective controls (0/0). Finally, several 24 h colchicine treatments in H28, such as 150/24, 300/24 for D1 and 150/24 for NA, had significantly higher percentages of induced calluses ($P < 0.05$) than their respective controls (0/0-D1 and 0/0-NA).

Regenerated plantlets were obtained from thirteen and fourteen out of fifteen different media for NRCV980385 and H28, respectively. The exceptions were the NA treatments supplemented with colchicine for 24 h (150/24-NA and 300/24-NA) in NRCV980385 and 0/0-D1 in H28. Plantlets regenerated from calluses were either albino or green, although some calluses were capable of regenerating both (Figs. 2, 3 for NRCV980385 and H28 respectively). There was a tendency for higher numbers of albino plantlets to be present when there was a high rate of plantlet regeneration. Albino plantlets regenerating calluses were the most

Table 3 Induced callus (IC) in all treatments assayed for the temperate japonica rice variety, NRVC980385, and the temperate japonica F2 hybrid, H28

	NRCV980385			H28		
	D1	D2	NA	D1	D2	NA
0/0	6.05	7.66	6.96	0.18	4.18	5.10
150/24	7.39	2.40*	0.00*	6.44*	2.73	8.67*
150/48	2.39*	3.51*	7.80	14.10*	17.73*	11.39*
300/24	5.23	3.44*	0.00*	10.55*	3.28	1.37*
300/48	5.81	5.12	8.56	18.75*	9.63*	12.54*

Values followed by * show significant differences at the 5% level in a Chi square test for homogeneity when compared with its hormone control colchicine-free medium (0/0)

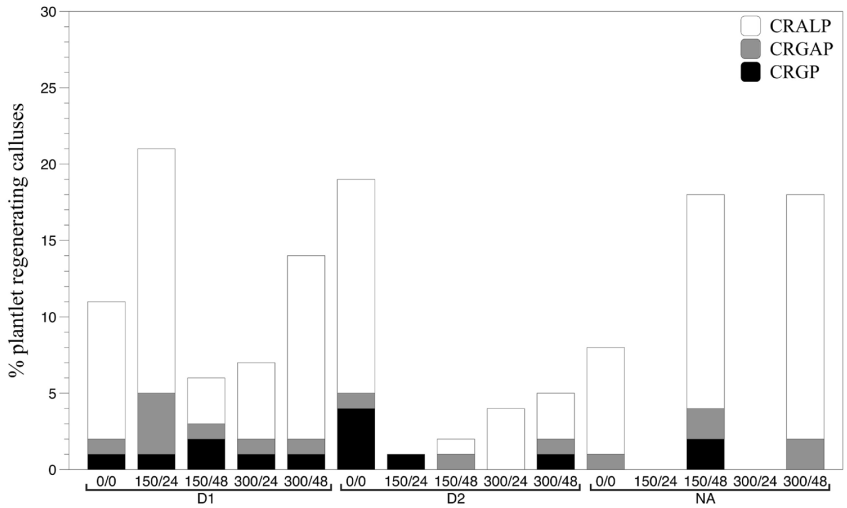

Fig. 2 Percentage of plantlets regenerating calluses for each induction media assayed in NRVC980385. *CRALP* callus regenerating albino plantlets, *CRGP* callus regenerating green plantlets, *CRGAP* callus regenerating green and albino plantlets

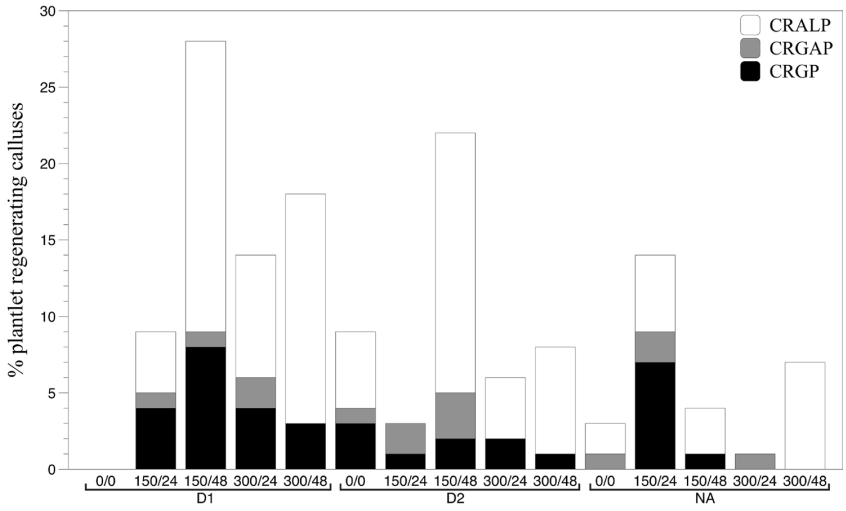

Fig. 3 Percentage of plantlets regenerating calluses for each induction media assayed in H28. *CRALP* callus regenerating albino plantlets, *CRGP* callus regenerating green plantlets, *CRGAP* callus regenerating green and albino plantlets

frequent, representing 77% of and 75% of NRCV980385 and H28 plantlets, respectively, when all media were grouped together.

NRCV980385 regenerated green plantlets in all D1 hormone media (1 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin). In contrast, under D2 hormone conditions (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin) the values for the total number of calluses regenerating plantlets were higher under colchicine-free conditions, with the number of green plantlets also being higher. Plantlets regenerated in the 150/24-D2 treatment were all green, unlike 300/24-D2 where all plantlets were albino. Finally, 0/0-NA and 300/48-NA conditions caused calluses to regenerate either albino plantlets or albino and green plantlets, but none of the calluses regenerated green plantlets alone.

The ability of H28 calluses to regenerate green plantlets was higher than NRCV980385 in almost all conditions (Fig. 3). Furthermore, colchicine treatments combined with D1 and NA hormone conditions displayed higher CRGP than their control treatments (0/0). Meanwhile, D2 hormone treatment without colchicine regenerated a higher number of green plantlets compared to colchicine treatments with this hormone, a tendency also observed for NRCV980385. Finally, 0/0-NA, 300/24-NA and 300/48-NA conditions had no calluses that regenerated green plantlets exclusively.

Effects of colchicine treatment on RDHP, G/A and RGDHP

The hormone factor has been grouped for both genotypes with the aim of analysing the colchicine effect on regenerated double haploid plantlets (RDHP), the green/albino plantlet ratio (G/A) and regenerated green double haploid plantlets (RGDHP) (Table 4). Values of RDHP and RGDHP for NRCV980385 were greater than for H28, but on average the G/A ratio was lower in NRCV980385 (Table 4). In addition, RDHP values for H28 in the four colchicine treatments at 48 h were higher than in the control, with both treatments showing significant differences.

Table 4 Regenerating double haploid plantlets per 100 anthers plated (RDHP), green/albino ratio (G/A) and regenerating green double haploid plantlets per 100 anthers plated (RGDHP) by genotypes according to colchicine treatment

Colchicine treatment	RDHP		G/A		RGDHP	
	NRCV 980385	H28	NRCV 980385	H28	NRCV 980385	H28
0/0	3.89	0.23	0.19	0.67	0.18	0.00
150/24	2.38	0.54	0.14	1.33	0.49*	0.22
150/48	1.77*	2.26*	0.28	0.55	0.22	0.29
300/24	1.80*	0.46	0.09	0.65	0.31	0.12
300/48	4.22	1.34*	0.12	0.20	0.75*	0.17

Three concentrations were assayed: 0 (control condition), 150 and 300 mg L^{-1}. For each colchicine concentration two exposure times, 24 and 48 h, were tested. In the end, 5 colchicine treatments were tested and named according to concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Hormone factors has been grouped. RDHP and RGDHP values followed by * are significantly different at the 5% level in a Chi square test for homogeneity in comparison with colchicine-free media (0/0)

On the most part, the G/A ratio for NRCV980385 and H28 was not significantly affected by the colchicine treatments. The one exception was H28 in the 150/24 colchicine treatment, which had a doubled G/A ratio in comparison to the colchicine-free control.

Calluses from all colchicine treatments regenerated green double haploid plantlets and the values were similar. The values for regenerated green double haploid plantlets (RGDHP) were always higher on colchicine treatments than on the colchicine-free treatment for both genotypes, with NRCV980385 having the highest values. For NRCV980385, the 150/24 and 300/48 colchicine treatments were significantly different ($P < 0.05$) from their 0/0 controls, with the number of regenerated green double haploid plantlets being 2.5 and 4 times higher respectively. For H28, the RGDHP values showed no significant differences when compared to its control due to the absence of regenerating green double haploid plantlets on 0/0. The best colchicine treatments for H28 were 150 mg L^{-1} at 24 and 48 h.

Haploid diploidization

Application of antimitotic agents to haploid plantlets obtained from the anther culture procedure hindered the rate of plantlet survival. After antimitotic treatment, most analysed plantlets were haploids (Table 5). Plantlets showing only double haploid ploidy were observed at the lower antimitotic concentrations (250 mg L^{-1} of colchicine and 1.25 mg L^{-1} of oryzalin). Within the plantlets that changed his chromosome content, mixiploids, including double haploid ploidy, were the majority. Moreover, when a higher antimitotic concentration was used the plantlet mortality rate increased, reaching 91.11% mortality with 1000 mg L^{-1} of colchicine. In the case of oryzalin, the mid-range concentration treatment (2.5 mg L^{-1}) showed the highest mortality, with a value of 34.78%. The percentage of plantlets that remained haploid after the treatment was higher when the antimitotic oryzalin was used (Table 5) at any concentration. The percentage of haploid plantlets and dead plantlets tended to increase with increases in the antimitotic concentrations.

Discussion

Anther culture is a powerful technique to produce rice DH plants. Nevertheless, the genotype effect is the major limiting factor, causing a differential response in callus induction as well as plantlet regeneration, ploidy and pigmentation previously reported by a number of authors (Mishra and Rao 2016; Herath et al. 2010; Khanna and Raina 1998; Raina and Zapata 1997; Moloney et al. 1989). Additionally, obtaining a high number of regenerating calluses is essential to increase the number of green double haploid plantlets displaying differential genotypes. Regenerated plantlets from the same callus or calluses from the same anther are more likely to be clones and therefore will have poor genetic variability. In addition, due to the fact that anther culture is a two-step process (i.e. initial development of calluses and subsequent regeneration of green plantlets from embryogenic calluses), researchers interested in obtaining new rice varieties from anther culture must avoid bottlenecks in the procedure. Low callus induction, low green plantlet regeneration and low double haploid regeneration can drastically limit the outcomes of anther culture. To minimize this, we proposed a workflow to study the response of the desired genotypes to anther culture over a six-month period using a range of induction media. Consequently, this study has focused on making preliminary assays to determine factors that could improve the yield of green double haploid plants. Additionally, the procedure has reported ways of reducing the amount of work to obtain DH plants in rice by: (i) reducing the time needed for the anther culture procedure, (ii) introducing a fast ploidy determination method, and (iii) a post anther culture diploidization protocol for rice.

Table 5 Post anther culture parameters of treated plantlets

Antimitotic	Concentration (mg L^{-1})	Post anther culture parameters					
		Total plants	% n	% 2n	% 2n mixploid	% mixploid	% dead
Control	0	20	100	0.00	0.00	0.00	0.00
Colchicine	250	47	38.30	2.13	29.79	2.13	27.66
	500	37	18.92	0.00	35.14	0.00	45.95
	1000	45	4.44	0.00	4.44	0.00	91.11
Oryzalin	1.25	31	77.42	6.45	3.23	0.00	12.90
	2.5	46	60.87	0.00	2.17	2.17	34.78
	5	47	70.21	0.00	6.38	0.00	23.40

Percentage n percentage of haploid plantlets with only a level of haploidy, *Percentage 2n* percentage of doubled haploid plantlets that with only a level of diploidy, *Percentage 2n mixiploid* percentage of double haploid plantlets with multiple ploidy levels, including 2n, *Percentage mixiploid* percentage of plantlets with multiple ploidy levels, excluding 2n

We included in our study the NRCV980385 genotype and the growth regulator 2,4-*D* in the induction medium at 2 mg L^{-1} in order to compare results with Serrat et al. (2014). In fact, the number of induced calluses was higher in our study than the previous one, which in turn resulted in a greater number of green double haploid plantlets. The cold pre-treatment was the main difference between the studies, which was changed from 7 to 10 °C and adjusted from a variable 7–12 days to a fixed 9 days. Another factor that could have affected the results is that in this experiment NRVC980385 genotype was a stabilized commercial genotype rather than a heterozygous seed batch as used previously in Serrat et al. (2014). Many authors have confirmed that cold pre-treatment has a stimulatory effect on androgenic response in several genotypes (Tian et al. 2015; Herath et al. 2010; Touraev et al. 2009). Moreover, a temperature of 10 °C is commonly used as a cold pre-treatment (Rukmini et al. 2013; Naik et al. 2017). Indeed, Naik et al. (2017) described that 7 days at 10 °C resulted in the best callus induction in a japonica cultivar. Therefore, the changes in cold pre-treatment enhanced the anther culture protocol with a higher rate of induced calluses.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-*D*) and naphthaleneacetic acid (NAA) are the most commonly used hormones for rice callus induction from anthers (Trejo-Tapia et al. 2002). The colchicine-free D2 treatment (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} of kinetin), induced the best culture conditions overall for both the NRVC980385 and H28 genotypes. These results are in agreement with many authors who have determined that 2 mg L^{-1} of 2,4-*D* results in the best culturability results for many genotypes (Chen et al. 2002; Herath et al. 2008). The effect of 2,4-*D* may be the promotion of rapid cell proliferation and formation of non-embryogenic callus as described for spring wheat (Ball et al. 1993). In addition, this auxin at this concentration is widely used in rice anther culture, although regularly combined with other auxins or other cytokines to obtain the best results (Serrat et al. 2014; Afza et al. 2000; Kaushal et al. 2014; Chen et al. 2002). In contrast, the effect of NAA, which is also commonly used in rice anther culture, may be to induce direct androgenesis (Yi et al. 2015; Alemanno and Guiderdoni 1994; Reiffers and Freire 1990). Finally, many authors use combinations of 2,4-*D* and NAA, to obtain better results (He et al. 2006; Xie et al. 1995). Nevertheless, our results do not show clear patterns of rice anther culturability between the two growth regulators.

A differential anther culture response was observed when adding colchicine to the induction medium, which depended on the genotype cultured. NRCV980385 callus induction was negatively affected by colchicine. On the other hand, H28 induced more calluses on colchicine-supplemented induction media. In maize, an increase in embryo frequency has been reported in the presence of colchicine (Barnabás et al. 1999; Obert and Barnabás 2004). In wheat, an absence of effect of colchicine on microspore embryogenesis has been reported by Barnabás et al. (1991), and a reduction was reported by Navarro-Alvarez et al. (1994). In the present study, 75% of the colchicine-supplemented induction media assayed for H28 had significant callus induction enhancement. Moreover, H28 callus induction increased in both colchicine concentrations at 48 h for all of the growth regulators assayed. This effect is also in accordance with Alemanno and Guiderdoni (1994), who described a significant (50%) increase in rice anther callusing with 500 mg L^{-1} of colchicine at 24 and 48 h.

The ability to regenerate plantlets, and specifically green plantlets, was different between the genotypes and the media assayed. NRCV980385 in colchicine-supplemented induction media had no positive effects on green plantlet regeneration. Indeed, treatments with 2,4-*D* at 1 mg L^{-1} regenerated as efficiently with colchicine-supplementation as the colchicine-free control. The ability of H28 to regenerate plantlets from calluses was greater than NRCV980385. At 1 mg L^{-1} of 2,4-*D*, colchicine increased the percentage of regenerating calluses, which was defined as the ability to regenerate higher numbers of green plantlets. Alemanno and Guiderdoni (1994) doubled the number of green plantlet-regenerating calluses with 250 mg L^{-1} of colchicine for 24 h relative to the colchicine-free treatment using the Miara genotype. In contrast, colchicine in the regenerating medium at 30 mg L^{-1} increased 7 times the number of green plantlets in comparison to the control in the Zao jing 26 genotype (Chen et al. 2002). Like these earlier reports, the number of calluses that regenerate green plantlets in H28 genotype, was increased. It is clear from the current work and previous reports that there is a strong effect of genotype on the outcome of anther culture.

In our study, the incidence of albinism was high for both genotypes. Albinism in plants is characterized by a lack of chlorophyll pigments and/or incomplete differentiation of chloroplast membranes in normally green tissues. Many studies have suggested that the use of colchicine in anther culture reduces the albinism ratio (Kumari et al. 2009; Barnabás et al. 1991; Ferrie et al. 2014). In our study, a reduction in albinism was only observed with 150 mg L^{-1} of colchicine during the 24 h treatment of the H28 genotype. This observation is in agreement with other authors who have noted no increases in the proportions of green plantlets after colchicine treatment at the callus stage (Hansen and Andersen 1998; Alemanno and Guiderdoni 1994). Furthermore, the number of double haploid regenerated plantlets also seems to be unaffected by colchicine treatments, irrespective of the different concentrations and exposure times tested. These results are in contrast with reports of an increase in regenerating double haploid plantlets when using colchicine (Barnabás et al. 1991). In our work, the

ploidy of green and albino plantlets was analysed to obtain the parameter of regenerated double haploid plantlets (RDHP), whereas other authors have usually only considered the regenerated green plantlets. Nevertheless, in our study, colchicine-supplemented media increased the proportion of regenerated green double haploid plantlets (RGDHP), which is in accordance with other studies (Alemanno and Guiderdoni 1994; Barnabás et al. 1991; Weber et al. 2005). Finally, all colchicine treatments yielded higher proportions of regenerated green double haploid plantlets for both genotypes in comparison to the colchicine-free induction media. In NRCV980385, the 150 mg L^{-1} colchicine treatment for 24 h and the 300 mg L^{-1} treatment for 48 h gave the best results ($P < 0.05$) compared to 0/0 control, and the numbers of RGDHPs were 2.5 and 4 times greater than the control, respectively. The H28 genotype was not able to regenerate green double haploid plantlets in colchicine-free induction media, and because of that a statistical test was not possible. This lack of green double haploid plantlets from colchicine-free media may be due to a low endoreduplication or low ability for endomitosis in H28, which entails a spontaneous duplication of chromosomes from the haploid (Chen and Chen 1980).

In both genotypes assayed, colchicine seemed to affect the endomitosis rate in the treated microspores. Endomitosis is described as nuclear chromosome doubling due to a failure of the spindle during metaphase (Kasha 2005). C-mitosis is a form of endomitosis caused by colchicine, which has the ability to abort mitosis and inhibit tubulin polymerization in animal and plant cells (Fltzgerald 1976; Pickett-Heaps 1967; Kasha 2005), and this explains the high yield of green double haploid plantlets in rice. Our observed 2.5-fold increase in the proportion of calluses regenerating diploid green plantlets is in accordance with the work of Alemanno and Guiderdoni (1994). Chen et al. (2002) also observed an increase in regenerated green double haploid plantlets when using regeneration media fortified with 75 mg L^{-1} colchicine, although higher concentrations caused harmful effects on calluses and regenerated plantlets. For this reason we assayed 300 and 150 mg L^{-1} concentrations to test lower concentrations than the 500 mg L^{-1} used by Alemanno and Guiderdoni (1994) and delimit the best colchicine concentration while avoiding toxicity. Finally, colchicine is widely used to increase the number of green double haploid plantlets in anther culture of other species and has had positive results in wheat (Hansen and Andersen 1998; Soriano et al. 2007), maize (Saisingtong et al. 1996), oats (Ferrie et al. 2014) and rapeseed (Mollers et al. 1994; Weber et al. 2005). Despite our results and those reported in the literature, we suggest that further studies should be performed in rice to investigate the ability of colchicine to increase the numbers of regenerated green double haploids above albino double haploids.

The main limitation of anther culture is the unknown interaction that occurs between media and genotypes. Our results describe completely different responses for callus induction, regenerating calluses, plant albinism and the ploidy of regenerated plantlets that are dependent on the growth regulator, their concentrations and the exposure time. Many authors have reported that the genotype affects the androgenic response (Lentini et al. 1997; He et al. 2006; Bagheri and Jelodar 2008) and that changes in medium composition can alter the response of different rice cultivars (Trejo-Tapia et al. 2002; Chen et al. 2002; Herath et al. 2008, 2010). However, manipulation of colchicine in induction media has not been reported previously for rice.

A complementary way to obtain DH plants is to perform a post anther procedure treatment of green haploid plantlets with antimitotics. It has been widely reported that antimitotic treatments of plantlets may change their ploidy (Chen et al. 2002; Ascough et al. 2008; Gallone et al. 2014; Sarathum et al. 2010; Omidbaigi et al. 2012; de Carvalho et al. 2005). In this study, the in vitro production of double haploid plantlets from already formed haploid plantlets was achieved. Most of the plantlets that survived were mixiploid with levels of diploidy, and it was from these latter plants that we were able to obtain double haploid seed from those tillers that were double haploids. Colchicine at 500 mg L^{-1} was the best in vitro treatment to double the ploidy (35.14% of plantlets treated). This concentration of colchicine was also used previously in an in vivo treatment of tillers with an effectiveness of 11.5% (Chen et al. 2002). Ascough et al. (2008) reported that when lower antimitotic concentrations were used the number of surviving plantlets was higher, but on the other hand the level of diploidization was also lower. Omidbaigi et al. 2012 reported that high concentrations of oryzalin did not have much effect on the survival ratio.

Anther culture in rice has been studied in many genotypes to achieve the best method of maximizing green double haploid plantlet formation through different stresses. Taking this earlier work into account, we selected a range of stresses to formulate protocols for two Mediterranean japonica rice genotypes that will form the basis of an anther culture procedure for a wide range of genotypes. The genotypes trialled each had specific responses to the experimental conditions. We have demonstrated that cold pre-treatment at 10 °C for 9 days increases callus induction. Without colchicine in the induction medium, we recommend 2,4-*D* at 2 mg L^{-1} and kinetin at 1 mg L^{-1} to obtain the highest values for callus induction and green double haploid plantlet regeneration for Mediterranean japonica rice varieties. Colchicine-supplemented induction media may increase the level of callus induction, depending on the genotype. Colchicine on the induction medium increases the green double haploid plantlet production in all treatments of concentration and time assayed. We have shown that post-anther culture colchicine

treatment at 500 mg L^{-1} increases the green double haploid production from green haploid plantlets. Our results highlight the importance of the genotype and media interactions effects on the anther culture efficiency in rice. This study stands out the necessity to continue studying the response of rice to anther culture to better understand the main mechanism of interaction between genotype and induction media.

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An improved anther culture procedure for obtaining new commercial Mediterranean temperate japonica rice (*Oryza sativa*) genotypes

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Short Communication

An improved anther culture procedure for obtaining new commercial Mediterranean temperate *japonica* rice (*Oryza sativa*) genotypes

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Abstract Rice is one of the greatest calorie supply for the world population, especially since its production is almost entirely destined to direct human consumption and its demand will increase along with the world population. There are efforts worldwide to increase rice yields by obtaining new improved and stabilized rice lines. The rice anther culture, a fast and cheap technique, allows to obtain double haploid lines in less than one year. We report its application with an improved protocol in four Mediterranean *japonica* rice genotypes at F_2 generation. We performed a screening test for cold-pretreatment at 5.0±0.1°C and concluded that the optimum duration was 9 days as it produced the higher rate of anther-derived *callus* induction. This revised protocol was successfully applied to the four genotypes, obtaining good results in all the procedure's steps. At the end, more than 100 of double haploid green plants were generated. Moreover, 9 lines obtained from the anther culture procedure showed good qualities for the Spanish market at the growing, farming and grain production level during the field assays. Therefore, we report an improved anther culture procedure for obtaining double haploid lines from temperate *japonica* rice genotypes showing high commercialization expectance.

Key words: anther culture, cold-pretreament, field assays, Mediterranean rice, *Oryza sativa*.

Rice is a highly important cereal cultivar in the world, with a total of 490.9 million tonnes (milled equivalent) produced in 2015/16 of which more than 80% was destined to direct human consumption (FAO Trade and Market Division 2017). In addition, it has been proposed that rice will be one of the main calorie supplies in the forthcoming years (FAO Rice Market Monitor 2016). Thus, there are efforts worldwide to accelerate the development of new rice varieties either to attain higher yielding rates and/or to obtain higher quality grains (Guimaraes 2009; Khush 2005; Moon et al. 2003; Peng et al. 2008; Zeng et al. 2017). Despite the efforts made, rice breeders' seed suffer recurrent deteriorations due to successive annual cultivation (IRRI 1988; Serrat et al. 2014). Programs for ensuring rice breeders' seeds stability are laborious and time-consuming (Briggs and Knowles 1967; Jennings et al. 1979; Serrat et al. 2014). In addition, selecting and stabilizing new rice lines from an F_1 cross is a long process that usually takes about 8 years minimum (Martínez et al. 1996; Serrat et al. 2014).

The anther culture technique, first developed in rice by Niizeki and Oono (1968), allows to obtain stabilized double haploid (DH) plants bypassing the inbreeding process. Moreover, it is the fastest method to obtain DH rice plants as can be performed in less than one year (Agache et al. 1989; Miah et al. 1985). Roughly, this technique is a two-step process from the initial development of *calli* to the subsequent regeneration of green plants from embryogenic *calli* (Mishra and Rao 2016). This technique has been used to obtain pure parental lines and to speed up descendant's selection after an artificial cross (Courtois 1993; Mishra and Rao 2016). Over the years, it has been shown that it is much easier to apply this technique on tropical *japonica* varieties, since they are more responsive at the *callus* formation and plant regeneration stages than Mediterranean *japonica* or *indica* varieties (Chen et al. 1986; Herath et al. 2007; Hu 1985; Miah et al. 1985; Mishra and Rao 2016; Serrat et al. 2014; Yan et al. 1996). Despite of that, we have previously reported an anther culture technique adaptation for a Mediterranean temperate *japonica* rice (*Oryza sativa*) cultivar (NRVC980385) to produce a new commercial cultivar (NRVC20110077; Serrat el al. (2014)), which however showed a very poor anther-derived *callus*

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induction.

Therefore, the main aim of this study is to test for the first time and improved anther culture procedure on F_2 rice genotypes coming from self-pollination of four crosses between different temperate Mediterranean *japonica* rice varieties. In addition, a secondary aim was to test the effect of a colder cold-pretreatment performed at different days of exposure for increasing the anther induction rate. This will allow to establish a standard and fast technique for obtaining commercial DH plants, with a high anther induction efficiency, from any temperate Mediterranean *japonica* rice line in development.

For testing this improved protocol, four different F_2 rice genotypes that resulted from self-pollination of an F_1 generation generated by crosses between Mediterranean temperate *japonica* rice cultivars were used (Table 1; germplasm rice genotypes were coded according to La Càmara cooperative seed producer simplified coding system). We employed the F_2 generation as characters segregation is maximum and plants will therefore provide high variability when obtaining the double haploid green plants (Guimaraes 2009). Plants were grown in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Barcelona, Spain) on four litre plastic containers filled with rice substrate as described in Serrat et al. (2014).

The anther culture procedure was performed similar to Serrat et al. (2014). The cold-pretreament was modified in order to enhance the anther-derived *callus* induction stage according to (Chen et al. 1986; Trejo-Tapia et al. 2002a, 2002b). We performed a screening test at 5.0±0.1°C during 8 to 12 days to select the best cold-pretreatment duration for using it for the anther protocol. Haploid *calli* spontaneously double their ploidy during the plantlet regeneration step, and thus develop into double haploid (DH) plants but could also develop into haploid, triploid or polyploid plants (Alemanno and Guiderdoni 1994). Further, the ploidy level was analysed with the aim of reducing greenhouse space and costs since haploid plants are sterile. The ploidy determination was performed by flow cytometry following the protocol described in Serrat et al. (2014). Dihaploid plants were cultured in greenhouse until seed-set, and seeds were harvested for the subsequent field assays.

For comparing the suitability of the improved anther protocol, several parameters were analysed in the four F_2 rice genotypes tested and NRCV980385 cultivar used in Serrat et al. (2014). These were: *callus* induction percentage (CI%)=number of anthers producing *calli*/number of plated anthers×100; *callus* production ratio (CP_{ratio})=number of produced *calli*/number of anthers producing *calli*; green plant percentage (GR%)=number of green plant regenerated/number of transferred *calli*×100; green double haploid plant percentage (GRDH%)=number of green DH plants regenerated/number of transferred *calli*×100. For comparing data among rice genotypes, we used two approximations: (i) visually, we calculated the confidence intervals (CI) using the following formula

$$CI = \% \pm 1.96 \times \sqrt[2]{\frac{\% \times (1 - \%)}{\text{number of observations}}}$$

and used them as a mean of standard error; and (ii) statistically, we performed a Chi-squared test with Yates correction (Zar 2010). No visual nor statistical approximations were used for CP_{ratio}, since due to its nature neither CI nor Chi-squared test with Yates correction were possible to calculate. Please note that due to the experimental procedure of the anther culture, we did not use replicates, thus total values of several parameters for each genotype assayed were used instead.

Finally, for testing rice genotypes with commercial interest, we performed a general field assay on 70 double haploid in order to screen overall diseases resistance and production estimates. Selected genotypes were assayed in small scale field assays in La Càmara experimental fields (Amposta, Tarragona, Spain). For this, two designs were used: (i) plant agronomical trait evaluation: 25 plants per genotype assayed were planted in row as to have 20 cm between each plant and 50 cm between rows; (ii) plant production evaluation: 80 plants per genotype assayed were planted in row as to have 20 cm between each plant and 25 cm between rows. Plant agronomical traits such as plant height (i.e., from the base of the plant to the top of the panicle), susceptibility to rice stem borers and

Table 1. Parental cultivar (P1 and P2) and F_2 rice genotypes produced by P1×P2 cross are listed using the simplified code system according to La Càmara seed producer. Anther culture in vitro results are show: number of plated anthers, number of anthers producing *calli*, number of *calli* generated, *callus* induction percentage (CI,%), *callus* production ratio (CP_{ratio}) and number of green double haploid plants regenerated in the four rice genotypes assayed. The error for CI(%) corresponds to the confidence interval.

Parental cultivar 1 (P1)[a]	Parental cultivar 2 (P2)[a]	F_2 rice genotype (P1×P2→F_1→F_2)	No of plated anthers	No of anther producing *calli*	No of produced *calli*	*Callus* induction (CI, %)	CP_{ratio}	No of green double haploid plants regenerated
rG3	NRCV980385 (rG0)	F_2-30	20185	27	160	0.133±0.050	5.93	30
rG4	NRCV980385 (rG0)	F_2-40	21301	99	547	0.465±0.091	5.53	70
rG4	rG2	F_2-42	18880	37	152	0.196±0.063	4.11	7
rG4	rG5	F_2-45	17456	72	360	0.412±0.095	5.00	17
—	—	NRVC980385[b]	42660	4	66	0.009±0.009	16.50	29

[a]rG: Rice genotype. [b]Data for NRVC980385 was obtained from supplementary material in Serrat et al. (2014).

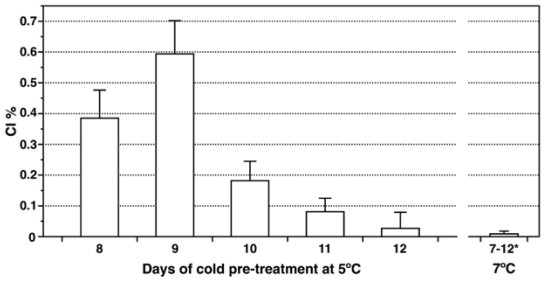

Figure 1. Effect of the cold pre-treatment on the callus induction percentage (CI,%) in the four rice lines assayed. The star (*) data on the right side of the graph corresponds to NRVC980385 retrieved from supplementary material in Serrat et al. (2014), in which the cold pre-treatment was performed at 7°C during 7–12 days. Error bars correspond to the confidence intervals.

resistance to blast and brown spot (Mew and Gonzales 2002), number of spikes per plant and inter-homogeneity (homogeneity between plants of the same genotype) were recorded 120 days after sowing. For plant production traits, the parameters evaluated were humidity (%) at the time of data recollection, 1,000-grains weight, percentage of whole grains (unshattered milled grains/total milled grains×100) and yield (kg of grains per hectare, kg/ha). NRVC980385 was used as a control to monitor field behaviour as it is a parental cultivar for F_2-30 and F_2-40, and is also common variety cultivated in the region (Català et al. 2007; Serrat et al. 2014).

Results of the cold-pretreatment duration test at 5.0±0.1°C as well as that performed at 7°C by Serrat et al. 2014 is shown in Figure 1. It was observed that for the *callus* induction (CI, %), there were significant differences between the duration in days of the cold-pretreament ($\chi^2_{Yates}(4)=94.0699$, $p<0.0001$; Figure 1), being 9 days the optimum for anther-derived *callus* induction. Moreover, it was also observed that a cold-pretreament at 5°C during 9 days instead of 7°C during 7–12 days had a higher CI% in all the days tested, being 0.254±0.072 in average and 0.009±0.009, respectively. Our results differ to those of Kaushal et al. (2014b) where the optimum is for 5 days at 12°C. These contrasting results can be explained by the fact that Kaushal et al. (2014b) used *indica* varieties, whereas the ones used in our experiment are Mediterranean temperate *japonica* genotypes. Trejo-Tapia et al. (2002a, 2002b) also studied the effect of cold-pretreament in anther-derived *callus* induction, but at 4°C. In their first study, 14 days was the best for the majority of the cultivars (tropical *japonica* sub-species) (Trejo-Tapia et al. 2002a), whereas in their second study 7 days was the best for H2500 cultivar (tropical *japonica* sub-species) (Trejo-Tapia et al. 2002b). Our cold-pretreament duration is situated between both works, suggesting that 9 days is an ideal time for this stage of the anther culture procedure to enhance the *callus* production in Mediterranean temperate rice *japonica* varieties. Moreover, the cold-pretreament at 5°C during 9 days radically increases the CI%, as the lowest CI% value reported in this study is almost 15 times higher than the one reported with NRCV980385 (Serrat et al. 2014).

Regarding the anther culture procedure for obtaining new rice lines, an average of approximately 19,500±1,600 anthers was plated for each of the four F_2 rice genotypes used in this experiment (details for each genotype in Table 1). *Calli* were produced from all of the four assessed genotypes differing in the CI%, but in average was higher than the one reported by Serrat et al. (2014). More in detail, it was observed that the genotype F_2-30, the one with the lowest CI%, had an anther-derived *callus* induction 12 times greater than NRCV980385 (Table 1). On the other hand, genotypes F_2-40 and F_2-45 displayed a CI% 51.7 and 45.8 times higher, respectively, when compared to NRCV980385. It is worth noting that the three of the F_2 genotypes in which the cultivar 4 was one of the used parental, yielded the highest CI%, being the higher the genotype F_2-40, cross between cultivar 4 and NRCV980385, with 0.465%. Statistical analysis showed significant differences between all the five rice genotypes (four F_2 rice genotypes and NRCV980385 cultivar) ($\chi^2_{Yates}(4)=193.9229$, $p<0.0001$). In the literature, CI% vary between as low as 0.2% to up to 77.9%, being the genotype the most important factor that determines these percentages (Bishnoi et al. 2000; Herath et al. 2007; Kaushal et al. 2014a, 2014b; Shahnewaz et al. 2004). Our CI% is situated in the lower ones, and it is probably due to the genotype of our F_2 rice genotypes, which do not favour *callus* formation as also observed in the study performed by Serrat et al. 2014. Several studies support this affirmation, since most of the differences can be explained by the genotype factor (Herath et al. 2007; Kaushal et al. 2014a; Khanna and Raina 1998; Shahnewaz et al. 2004; Yan et al. 1996). Despite the low CI% reported in this study when compared to the majority of the literature, the number of *callus* obtained was higher than that reported by other authors (Shahnewaz et al. 2003, 2004).

The higher anther-derived *callus* induction translated in a higher number of *calli*, 1,219 in total for the four F_2 genotypes assayed (Table 1). Moreover, the number of *calli* produced was much higher in this study compared to NRVC980385. The *callus* production ratio (CP_{ratio}) was similar in the four rice genotypes, being F_2-30 and F_2-40 the ones that showed higher values of 5.9 and 5.5, respectively. In average, the CP_{ratio} only corresponded to 31.2% of the displayed by NRVC980385 (Table 1) though we expected, as seen for CI%, that this parameter would be higher. This can be explained by considering the following: (i) average of anthers producing *callus* for our experiment was 59±17 whereas for Serrat et al. (2014) only four anthers were used for *callus* production; (ii)

average of *callus* produced were 305±94 and 66 in our experiment and in Serrat et al. (2014), respectively. Thus, although having in average a low CP_{ratio} in our study, we expect to have a higher chromosomal variability of the rice genotypes since an elevated number of *calli* coming from a larger number of anthers was obtained. It is worth nothing that not much data is available in the literature regarding CP_{ratio} which in turn does not allow for much comparison. Nevertheless, it is of high importance since it gives information if regenerated plants come from several or few *calli*, thus we propose that this ratio should be regularly given.

The green plant percentage (GR,%) was in average 89.6±5.9% among the four rice genotypes tested as seen in Figure 2, which is similar to our prior results using NRVC980385. But, it is noteworthy that the GR% was considerably higher than in other articles (ranging from 2 to even 16 times more) (Herath et al. 2007; Kaushal et al. 2014a, 2014b; Shahnewaz et al. 2003, 2004; Trejo-Tapia et al. 2002a, 2002b). Statistical analysis also showed that there are significant differences between the 5 rice genotypes ($\chi^2_{Yates}(4)=6.3447$, $p=0.1478$; Figure 2).

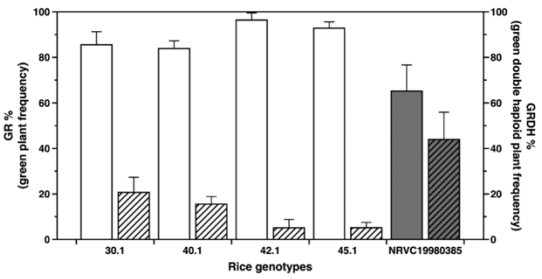

Figure 2. Total percentage of green regenerated plants (GR%) and total percentage of green double haploid regenerated plants (GRDH%) in the four rice lines assayed (white bars). Data for NRVC980385 was retrieved from supplementary material in Serrat et al. (2014) (grey bars). Solid bars correspond to GR% and hatched bars to GRDH%. Error bars correspond to the confidence intervals.

The green double haploid plant percentage (GRDH,%) reported in this study ranges from 11.5 to 47.1% of that reported with NRVC980385, being the genotypes F_2-30 and F_2-40 the ones displaying the higher GRDH% among our four F_2 rice genotypes (20.7 and 15.5%, respectively). In this study, more than 75 plants for each genotype were analysed, whereas in Serrat et al. (2014) only 43 in total were analysed. The GRDH% was significantly different among the 5 varieties ($\chi^2_{Yates}(4)=67.6942$, $p<0.0001$; Figure 2). This parameter is of the uttermost importance since those plants are viable and suited for field assay evaluations, which is the final purpose of this procedure. No data for this is available in the literature thus we cannot further compare. The last stage of the process was to acclimatize in vitro plants to greenhouse conditions in rice substrate. We transplanted a total of 547 green double haploid plants produced from the 4 F_2 genotypes, of which in average 89±4% were successfully grown to maturity (data not shown), which is greater than the 67±8% in average that is reported by Herath et al. (2007). Similarly, only 12±8% of the total of the transplanted plants showed more than 5% of sterility (data not shown), which is a better success rate than 24±31%, value observed in the work by Herath et al. (2007).

During the general field assay of 70 double haploid (DH) lines, it was observed that all DH plants coming from F_2-42 and F_2-45 along with some DH plants of F_2-30 and F_2-40 lacked agronomic and commercial interest (data not shown). Therefore, a total of 9 lines were selected for agronomical and production traits, which showed high inter-homogeneity and a high tillering activity (more than 40 tillers per plant; data not shown). The average height of the lines was 70.7±2.2 cm, which is in the range of those cultivated in the Ebro Delta. Moreover, 7 of them were shorter than Gleva, the shortest cultivated variety in the region (Pla et al. 2017). Overall, all evaluated lines showed a high

Table 2. Agronomical and production traits evaluation for the lines assayed in the field assays.

Anther-derived rice line	Height (cm)[a]	Plant agronomical traits evaluation			Plant production traits evaluation[d]			
		Fungal disease resistance[b]		Rice stem borers resistance[b,c]	Humidity (%)	1,000-grain weight (g)	Whole grains (%)	Yield (kg/ha)
		Blast[c]	Brown spot[c]					
F_2-30.C1	68.5±1.9	+++	+++	++	13.8	27.5	67.3	5740
F_2-30.C2	70.5±0.6	+++	+++	++	14.0	28.8	66.7	5828
F_2-40.C15	66.3±4.1	+++	+++	++	13.9	32.5	67.8	11393
F_2-40.D37	68.5±1.3	+++	+++	+	13.8	35.0	63.3	11598
F_2-40.D39	62.8±2.1	+++	+++	+++	13.6	35.0	61.4	10338
F_2-40.D118	65.3±0.5	+++	+++	+	13.8	35.0	66.9	11328
F_2-40.D173	59.0±2.7	+++	+++	++	13.9	30.0	63.0	11670
F_2-40.D174	61.5±1.3	+++	+++	++	13.9	32.5	65.9	11760
F_2-40.D266	90.3±4.4	+++	+	−	14.1	35.0	63.9	13553
NRVC980385	94.0±2.7	+++	+++	+	14.2	27.5	65.3	11325

[a] The value shown correspond to the mean of 25 plants and the SD. [b] Resistance scale is the following: −: sensible; +: low resistance; ++: medium resistance; +++: high resistance. [c] Blast and brown spot diseases are caused by *Magnaporthe oryzae* and *Helminthosporium* sp. respectively, and rice stem borers by *Chillo suppresalils*. [d] Production traits evaluation data for NRVC980385 was recorded at the same time of the F_2 rice lines assayed.

resistance to fungal diseases and medium resistance to rice stem borers (Table 2), except for F_2-40.D266 plants which was promising in terms of production during the general field assay. Regarding blast resistance, it was in general higher in comparison to the varieties regularly cultivated in the region of the Ebro Delta (Català et al. 2009; Pla et al. 2017). No literature was available for comparing tolerance to brown spot disease and the rice stem borers in local field conditions. Despite this, resistance to brown spot was similar to that of the control variety (NRVC980385), and rice stem borers resistance was higher than NRVC980835 for several lines. The 1,000-grain weight (determined by grain length, width and thickness) of the lines was in average 31.6 g, comparable among them since the humidity range was 13.6–14.2%. Of the assayed lines, all of them with the exception of F_2-30.C1, as seen in Table 2, showed a 1,000-grain weight higher than NRVC980385 and other *indica* and *japonica* varieties (Fan et al. 2006; Koutroubas and Ntanos 2003). The whole grains percentage was variable among the 9 lines assayed but ranged between 60–70%, similar to NRVC980385 and several other Spanish varieties values (Català et al. 2009), thus these lines are suitable for large scale production. In terms of yield, lines of the F_2-30 genotype were below those reported for Gleva (most cultivated variety in the region) and NRVC980835 cultivar (Pla et al. 2017), and half of the values reported for F_2-40 genotype lines (Table 2). On the other hand, lines of the F_2-40 genotype displayed higher yields than Gleva (Pla et al. 2017). Furthermore, the observation that F_2-40.D266 was promising in terms of production was certain as its yield was the highest among the lines tested and higher than the Spanish rice varieties including the NRVC980835 cultivar (Pla et al. 2017). Nevertheless, to fully characterize and evaluate the assayed lines, direct seeded field assays should be performed in a medium (and maybe even large scale) in order to better assess for pathogens resistance, plant height and yield.

In conclusion, we have shown that the improved anther culture protocol can be successfully applied in different F_2 rice genotypes between temperate *japonica* rice genotypes to obtain green double haploid plants. Moreover, we have observed that genotype is one of the main factors that affects the anther culture protocol success. Despite this, we have determined that the cold-pretreatment improvement, 9 days at 5.0±0.1°C, greatly increases the anther-derived *callus* induction in temperate *japonica* Mediterranean rice crossed genotypes at the F_2 generation, since the number of green double haploid plants obtained at the end of the anther culture procedure was high. Furthermore, 7 of the 9 lines evaluated in the field showed good qualities at the agricultural and production level. Therefore, these varieties are suited to be submitted to direct seeded medium scale assays before registry for their subsequent commercialization. Thus, in conclusion, our proposed method for Mediterranean *japonica* rice is highly applicable to rice genotypes at the F_2 generation of different *japonica* rice cultivars for producing new lines that could be registered and commercialized as new varieties.

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Conflicts of interest

The authors declare that they have no competing interests.

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Nuclei Release Methods Comparison for Fresh Leaves of Rice (*Oryza sativa*) for Efficient High Throughput Flow Cytometry Ploidy Studies

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Abstract

Flow cytometry trituration methods and the efficiency of isolation buffer solutions are compared in this study for extraction of nuclei from fresh leaves of rice. The razor blade sample trituration procedure has been widely used to release nuclei from tissues in many plant species, and combined with different isolation buffers for low throughput analysis. In contrast, the bead beating trituration method has rarely been used for DNA ploidy determination, despite it being proposed as a less tedious alternative procedure to prepare nuclear suspensions. In this study, bead beating was assessed and compared with the traditional chopping procedure. Each trituration method was combined with one of three nuclear isolation buffers (i.e. Hanson's, Otto's and LB01 buffer). Bead beating was applied for the first time using all three of the buffers, resulting in a rapid and effective procedure for ploidy determination in fresh rice leaves. In addition, bead beating saved, while reducing the exposure of the user to harmful substances. The best results were obtained when Hanson's nuclear isolation buffer was combined with the bead beating trituration method.

Keywords: flow cytometry, plant breeding, bead beating, chopping, fresh leaves, *Oryza sativa*, doubled haploid, anther culture

1. Introduction

Flow cytometry (FCM) is an analytical technique that allows multiparametric analysis to be performed on any stained particle sized between 0.2 and 150 μm. The FCM technique was first developed in the 1950s to count blood cells (João et al., 2006b). Currently, FCM is widely applied to perform different types of analysis in diverse areas of investigation (Langerhuus et al., 2012; Trend et al., 2015; Zedek et al., 2016). Nevertheless, it was not until the late 1980s that FCM became an important technique in plant research, nuclear DNA content estimations, cell cycle analysis and ploidy determination. The delay in applying FCM in plant science was due to technical problems in obtaining intact nuclear preparations from tissues with rigid cell walls (João et al., 2006a). Estimating ploidy in intact plant cells was unsuitable due to cell wall auto-fluorescence and disturbances to the fluid stream caused by the irregular shape of plant cells. For these reasons, the first reported successful FCM analysis in plants used hydrolytic enzymes to digest cell walls followed by release of the nuclei from fixed preparations.

Galbraith et al., (1983) devised a trituration method in which a suspension of intact nuclei was easily obtained by chopping a small amount of fresh tissue with a razor blade in a suitable isolation buffer. Since then, nuclear isolation via razor blade trituration of plant tissues has been widely used by many authors (Arumuganathan & Earle, 1991; De Laat et al., 1987; Doležel et al., 1998; Doležel et al., 1989; J.; Doležel & Göhde, 1995; Hanson et al., 2005; Loureiro et al., 2006a; Miyabayashi et al., 2007; Uozu et al., 1997). However, when the chopping trituration procedure is used, each sample needs to be prepared individually, thus being both time consuming and low throughput. In contrast, an alternative procedure known as bead beating is faster for plant ploidy determinations using FCM. Hanson et al. (2005) showed how samples could be mechanically ground with beads to expose the nuclei and easily extract them with an isolation buffer. Although bead beating has been used in FCM by some authors (Cousin et al. 2009; Roberts, 2007), razor chopping is still the predominant trituration

procedure for preparing nuclear suspensions. Nevertheless, Cousin et al. (2009) demonstrated the simplicity and efficiency of measuring 192 plant samples within 6 h via a bead beating procedure to isolate nuclei for FCM.

Alongside these two nuclear extraction procedures, numerous isolation buffers and staining procedures have also been published for FCM in plants. No less than twenty-five nuclear isolation buffers have been developed, including Galbraith's buffer (Galbraith et al., 1983), Hanson's buffer (Hanson et al., 2005), Arumuganathan and Earle's buffer (Arumuganathan & Earle, 1991), LB01 (Doležel et al., 1989) Tris-$MgCl_2$ (Pfosser et al., 1995), Marie's buffer (Marie & Brown, 1993) and Otto's buffers (Otto, 1990) as the most popular ones.

FCM is a necessary step in plant breeding when double haploids are desired (Hooghvorst et al., 2018). In rice (*Oryza sativa*), some authors have used the chopping procedure at different stages in the life cycle, such as the seed stage (Miyabayashi et al., 2007; Uozu et al., 1997) or with young plantlets (Meister, 2005). However, the bead beating procedure has not been used widely to extract intact nuclei and determine ploidy levels, except in *Brassica napus*, *Allium cepa*, *Nicotiana tabacum*, *Petroselinum crispum*, *Rosa canina* and *R. rugosa* (Cousin et al., 2009; Roberts, 2007). Furthermore, only Hanson's buffer has been reported as being used during the bead beating trituration method.

The aim of this study is to determine the simplest and most efficient trituration procedures and isolation buffer combinations for rice (*O. sativa*) ploidy analysis. We report here a comparison between bead beating and the chopping trituration methods, and the use of three different isolation buffers previously used to estimate the DNA ploidy level of plants using haploid, diploid and triploid anther culture derived lines of rice. It is concluded that the three nuclear isolation buffers can be used with bead beating trituration, but the best results were obtained when Hanson's nuclear isolation buffer was used.

2. Materials and Methods

2.1 Preparation of Nuclear Samples

About 20 mg of fresh young leaves from 3-4-week-old plantlets regenerated from anther culture were used to carry out the study. Leaf samples were obtained in sterile conditions and weighed on a precision scale. When samples were chopped, leaves were placed on a 100 x 15 mm polystyrene Petri dish, containing 1 mL of the appropriate isolation buffer (Hanson's buffer, Otto's buffers and LB01, see Table 1). Samples were chopped for 2-3 minutes using a sharp double-edged razor blade. When bead beating trituration was used, the leaf samples were compacted into spherical shapes and then added to 2 mL microcentrifuge tubes (Fisher Scientific, Pennsylvania, USA) each containing a steel bead. To each microcentrifuge tube, 300 µL of the nuclear isolation buffer was added. Samples were shaken for 48 seconds at 25 Hz in a 400 MM TissueLyser (Retsch, Mettmann, Germany). The suspension obtained was homogenized by pipetting up and down several times.

Table 1. Nuclear isolation buffers and their chemical composition. The used staining solutions were also specified for each isolation buffer

Buffer	Composition
Hanson's [Hanson et al. 2005] (Hanson et al. 2005)	*Isolation buffer*: 0.1 M citric acid; 0.5% Triton X-100. *Staining solution buffer*: 11.36 g Na_2HPO_4; 12 mL PI stock (1 mg/mL); 20 mL 10x stock (100 mM sodium citrate, 250 mM sodium sulfate) in 200 mL of distilled water.
LB01 [Doležel et al. 1989] (J.; Doležel, Binarova, and Lucretti 1989)	*Isolation buffer:* 15 mM TRIS; 2 mM Na_2EDTA; 0.5 mM spermine·4HCl; 80 mM KCl; 20 mM NaCl; 15 mM β-mercaptoethanol; 0.1% (v/v) Triton X-100; pH 7.5. *Staining solution:* PI (50 µg/mL).
Otto's [Otto 1990] (Otto 1990)	*Isolation buffers:* - Otto I: 100 mM citric acid; 0.5% Tween 20. - Otto II: 400 mM $Na_2HPO_4·12H_2O$. *Staining solution:* PI (50 µg/mL).

The homogenates were then filtered through 33 µm nylon mesh into round-bottomed polystyrene 12x75 mm test tubes. When Otto's buffers were used, Otto II isolation buffer was added to the nuclear suspension in the test tube at a 1:4 proportion (buffer: suspension).

After nuclei extraction with any of the isolation buffers, RNase (50 µg/mL) was added to each test tube. Twenty minutes later, PI (50 $\mu g \cdot mL^{-1}$) was added when using LB01 or Otto's isolation buffer, while the staining solution specific to Hanson's buffer was added in a 1:2 proportion (stain:buffer). Samples were incubated for fifteen minutes on ice and in the dark before analysis, shaking the test tube occasionally.

2.2 Flow Cytometry

Samples were analyzed by using a Gallios™ flow cytometer (Beckman Coulter, Indianapolis, USA) provided by the *Centres Científics i Tecnològics* of the *Universitat de Barcelona* (CCiTUB). This cytometer was equipped with a high-throughput sampler and four different lasers: blue solid-state diode (488 nm), yellow solid-state diode (561 nm), red solid-state diode (638 nm) and violet solid-state diode (405 nm). Nuclear PI fluorescence was measured using the 488 nm laser.

Samples were run at a low flow rate (10 $\mu L \cdot min^{-1}$) and this flow was kept constant throughout the experiment. In every sample 5,000 nuclear signals were analyzed during a maximum of 600 seconds. To ensure that all 5,000 of the nuclei were detected in each sample, two delimiting conditions were established. Both delimiting areas were defined by analyzing diploid samples prepared with chopping and bead beating procedures. Only events or particles inside both the FSC (forward-scattered light dispersion, proportional to the area or size) and SSC (side-scattered light dispersion, proportional to the internal complexity) delimiting fluorescence intensity areas, as defined with control samples, were considered nuclei. Sample data were acquired using Summit Software v4.3 (Cytomation, Colorado, USA).

2.3 Experimental Design and Statistics

During this study, nine samples were prepared for each experimental condition. Six different methodologies were applied to obtain a suitable nuclear suspension, assessing three different isolation buffers and two different trituration procedures. The three nuclear isolation buffers used were: (i) Hanson's buffer (Hanson et al., 2005), (ii) LB01 buffer (Doležel et al., 1989) and (iii) Otto's buffers (Otto, 1990). Two of these buffers were applied in a one-step protocol (Hanson's and LB01) while the last one (Otto's) was used as a two-step protocol. In the case of Hanson's nuclear isolation buffer, the staining solution used to label nuclei was a buffer described by the same author containing propidium iodide (PI). Because the stain was crucial for assessing ploidy, PI was also selected for the other buffers.

With the aim of making a complete comparison between nuclear extraction procedures and isolation buffers in each procedure, the following parameters were recorded: time (minutes) needed for sample preparation taking into account the time invested in sample collection, development of the relevant protocol and run time of the flow cytometer for ploidy determination; the half peak coefficient of variation (HP-CV (%)) of the resulting peak, this parameter estimating nuclear integrity and DNA staining variation; debris background factor (DF (%)) of the nuclear suspension; nuclear yield factor (YF ($nuclei \cdot s^{-1} \cdot mg^{-1}$)) which calculated the amount of nuclei in suspension. Statistical analyses were carried out using Welch's ANOVA with Statgraphics Centurion XVII (Statpoint Technologies, Inc. Warrenton, Virginia, USA). All the data were analyzed at the 95% confidence level.

3. Results

3.1 Ploidy Determination

DNA content and plant ploidy were measured in both trituration methods with the three isolation buffers and in the different ploidy plants. Reference peaks from the chopping or bead beating procedures were detected in different channels. Thus, diploid plants showed a reference peak around channel 250 with the chopping procedures, and 280 when using the bead beating procedures. Moreover, in both trituration procedures, the reference peaks of the haploid samples correlated to half the value of their diploid counterparts, with 125 in the chopping procedures and 140 in the bead beating procedures (Fig. 1). Triploid samples were also found around channels 375 and 420 when using the chopping and bead beating procedures, respectively.

The samples prepared using the chopping procedure did not have any type of irregularity in the PI fluorescence reading along the time axis. However, obstructions in the injection needle were observed for bead beating samples. As a consequence, large numbers of particles were injected suddenly through the needle when the obstruction was cleared, resulting in a linear PI intensity dispersion. These obstructions appeared equally in every sample when using bead beating, independent of the isolation buffer used.

3.2 Trituration Method

Sample trituration using the chopping procedure was slow and tedious compared to bead beating. The bead beating method was faster than chopping, taking from 8.39 to 11.52 and 26.49 to 32.83 minutes, respectively (Table 2). The step that highly reduced was the automatic trituration procedure in bead beating. The HP-CV parameter showed no significant differences between the sample trituration procedures ($P>0.05$). The HP-CV average was 5.10% for the chopping procedure and 5.08% for the bead beating procedure.

Significant differences between both trituration procedures were found ($P\leq0.05$) for the DF. The DF was

significantly higher in samples prepared with bead beating with an average of 47.67% compared to 33.27% with chopping (Table 2). Significant differences between trituration procedures (P≤0.001) were also observed in the YF. The average YF observed with the chopping procedure was 0.82, while the average calculated from bead beating was 3.16 nuclei·s^{-1}·mg^{-1}.

Table 2. Mean and SD values for half peak coefficient of variation (HP-CV) percentage (%), time analysis (s), debris background factor (DF) percentage (%), nuclear yield factor (YF) (nuclei·$s^{-1}mg^{-1}$) values, and consumed time (min) per sample in relation to the methodology assayed: chopping and bead beating trituration technique combined with three buffers assayed, Hanson, Otto and LB01

Method	HP-CV (%)	Time for analysis (s)	DF (%)	YF (nuclei·$s^{-1}mg^{-1}$)	Time (min)
Chopping					
Hanson	4.77 ± 1.05	188.51 ± 29.19	58.19 ± 25.46	1.28 ± 0.48	26.49
Otto	6.33 ± 1.47	561.23 ± 102.93	15.60 ± 5.02	0.30 ± 0.14	32.83
LB01	3.28 ± 0.70	373.51 ± 182.27	26.03 ± 5.98	0.87 ± 0.50	29.01
Bead Beating					
Hanson	4.05 ± 1.37	208.64 ± 116.64	42.31 ± 6.04	2.65 ± 1.34	9.98
Otto	3.78 ± 1.58	308.10 ± 120.08	42.17 ± 7.00	1.67 ± 0.77	11.52
LB01	4.69 ± 1.51	114.09 ± 48.78	58.53 ± 7.47	5.16 ± 2.49	8.39
Significance[a] in F tests					
Among trituration procedures	ns	**	*	***	
Among Buffers- Chopping	***	***	**	**	
Among Buffers-BB	ns	**	**	*	

[a]ns, not significant (P>0.05); * P<0.05; **P<0.01; ***P<0.001.

3.3 Isolation Buffer

Sample preparation time was similar when using Hanson's and LB01 buffers, but longer when using Otto's buffers due to the Otto I and Otto II components resulting in a two-step protocol. Time per sample was significantly different between buffers (Table 2), with Otto's buffers in particular requiring 2 to 3-fold more time compared to the other buffers (P≤0.01).

The average HP-CV (%) was not significantly different (P>0.05) between buffers when bead beating was applied to triturate samples (Table 2). In contrast, the average HP-CV (%) showed significant differences between the three buffers (P≤0.001) under the chopping procedure. The HP-CV (%) value for LB01 buffer was 3.28%, which was lower than the values observed in Hanson's and Otto's buffers, which were 4.77% and 6.33% respectively.

Although both trituration methods showed significant differences between buffers in terms of DF (P≤0.05), the lowest debris values were obtained when using Otto's buffers in both of trituration methods.

Significant differences were also found between the trituration methods for YF (P≤0.05, Table 2). Otto's buffers showed the lowest YF average in both trituration methods, being significantly lower than the YF average observed using Hanson's or LB01 isolation buffers.

4. Discussion

Flow cytometry has been used to determine ploidy in plant species since development of this analysis technique. Many isolation buffers have been described for many species, and in 2007 bead beating was introduced as a new trituration method (Roberts, 2007). To assess the viability of adapting bead beating to ploidy analysis in rice we have used a selection of isolation buffers and compared the two known trituration methods. Our results highlight the efficiency and efficacy of the bead beating method. This work shows that when using bead beating trituration there is the possibility of using LB01 and Otto's buffers apart from Hanson buffer, which has been previously used. Clearly, LB01 was the best isolation buffer when the chopping procedure was used for nuclear isolation. On the other hand, Hanson's buffer was the most suitable isolation buffer when bead beating was assessed.

It was possible to determine rice ploidy levels from fresh leaf samples following both the bead beating and chopping trituration procedures. The traditional chopping procedure was expected to work satisfactorily in rice because it has been widely used by other authors (Miyabayashi et al., 2007; Uozu et al., 1997). Our results highlight the usefulness of bead beating method in haploid and double haploid plantlet determinations as shown by Cousin et al. (2009). In addition, our results show that bead beating is a suitable method for preparing nuclear

suspensions from fresh rice leaves not only using Hanson's buffers, but also LB01 and Otto's buffers.

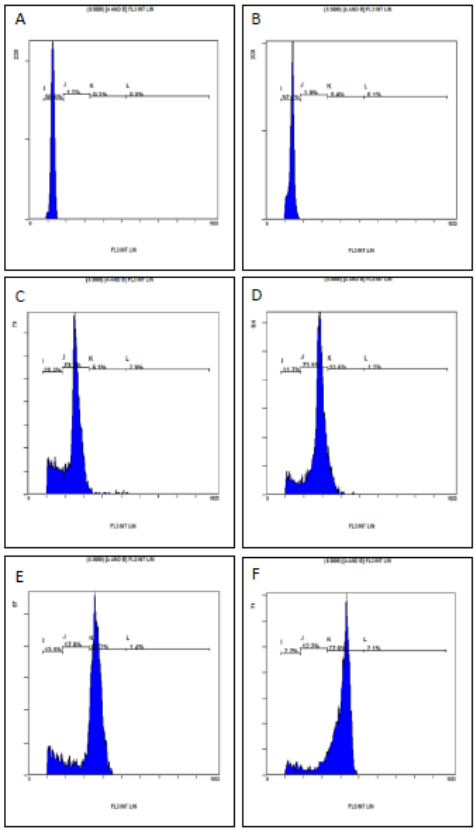

Figure 1. Representative propidium iodide fluorescent histograms corresponding to G_0/G_1 peak of analyzed haploid (A, B), diploid (C, D) and triploid samples using chopping (A, C, E) or beat beating (B, D, F). Peaks were represented from 0 to 1023 channels (X axis) and the number of nuclei corresponding to G_0/G_1 found in each of the channel (Y axis). (A) haploid sample triturated with chopping procedure showed G_0/G_1 peak at channel 125. (B) haploid sample triturated with bead beating procedure showed G_0/G_1 peak at channel 140. (C) diploid sample triturated with chopping procedure showed G_0/G_1 peak at channel 250. (D) diploid sample triturated with bead beating procedure showed G_0/G_1 peak at channel 280. (E) triploid sample triturated with chopping procedure showed G_0/G_1 peak at channel 375. (F) triploid sample triturated with bead beating procedure showed G_0/G_1 peak at channel 420

The half peak coefficient of variation (HP-CV) of the DNA peaks was of major importance to determine the quality of the nuclear suspensions (Doležel et al., 1989; Loureiro et al., 2006a; Taylor & Milthorpe, 1980). DNA peaks with an HP-CV≤5.0% are considered acceptable, while an HP-CV≤3.0% is considered excellent (Galbraith et al., 1983). The HP-CVs were not significantly different ($P>0.05$) between the trituration procedures, so the nuclear quality and integrity was unaffected by the trituration method, as pointed by (Roberts, 2007). Bead beating trituration procedures provided average results of around 5.0%, indicating quite good nuclear integrity, and this was independent of the nuclear isolation buffer used. Roberts (2007) obtained HP-CV values lower than 4% using bead beating in *R. rugosa*, suggesting that nuclear quality values can be improved depending on the methodology or the species.

The histogram peak positions of haploid, diploid and triploid samples in the cytometer channel ranged the number depending on the trituration method used. Bead beating samples had higher peak channel positions than the chopped samples. This channel displacement could be explained by the fact that the bead beating procedure resulted in rougher nuclear extraction with high amounts of debris present. Consequently, broken or incomplete chloroplasts containing chlorophylls and other subcellular tissue fragments could affect this peak displacement.

Indeed, the strong red and/or orange auto-fluorescence (>600 nm) of chlorophylls can cause substantial interference with the PI-based quantitative signal emitted from genetic material, as stated by Hyka et al. (2013). In addition, the same tissue fragments could also have been responsible for the needle obstructions during injection of bead beating samples, contributing to irregular PI fluorescence analyses. Both of the irregularities in the PI fluorescence, which is considered proportional to the amount of genetic material, may have altered the main peak positions of the bead beating samples. Fluorescence interactions from chlorophylls and cytosolic debris have also been reported to affect PI fluorescence by Noirot et al. (2003) and Loureiro et al. (2006a). A correlation between HP-CV and DF was observed by Emshwiller (2002). However, in subsequent studies this correlation was only found in some species, and was not confirmed in many others (Loureiro et al., 2006a). In this study, no correlation was observed between CV and DF and this could be due to the significantly larger amounts of debris found in samples triturated using bead beating, corroborating that chlorophylls and tissue remnants in suspension could affect the fluorescence analyses of bead beating samples and resulting in a channel number displacement. In the same way, the nuclear yield factor values (YF) were significantly higher in bead beating samples than the chopped ones, which indicated greater nuclear extraction and nuclear concentration in these samples. Consequently, bead beating yielded acceptable quality suspensions, obtaining similar HP-CV and higher YF averages than the chopping trituration procedure.

Bead beating minimized the sample preparation costs by reducing the required sample preparation time. Cousin et al. 2009 described a similar procedure using 96-well flow cytometer racks. Furthermore, it is important to consider that when using the traditional chopping procedure, the operator is in a closer contact with isolation buffers and some harmful substances such as β-mercaptoethanol (Roberts, 2007) than when using bead beating. Thus, the semi-automated bead beating procedure avoids direct contact with toxic reagents.

Nonetheless, the described protocols could be further improved by centrifugation of the nuclear suspension and resuspending the pellet in a smaller volume of isolation buffer. This way, higher YF values could be obtained, although it would increase the sample preparation time and costs. Surprisingly, the main quality parameters obtained from bead beating samples were even better than those observed from chopping samples except for the DF parameter. Although no determining higher number of broken nuclei, it would be advisable to reduce background debris in bead beating samples to obtain even higher quality suspensions in the future.

Years ago, considering the diversity in tissue anatomy and chemistry in plant species, it was thought that no single nuclear isolation buffer would be applicable for nuclear isolation for ploidy determination across all species (Jaroslav et al. 2005). Later quantitative data suggested this was true, and that none of the most popular nuclear isolation buffers worked well with different species (Loureiro et al., 2006a). Therefore, it was important to test different nuclear isolation buffers so that the FCM technique for ploidy determination in rice could be optimized. Our results demonstrated that the ploidy level may be determined in rice by using any of the three tested nuclear isolation buffers. Our data shows a different response of buffers depending on the trituration procedure selected and the suitability of bead beating to perform high quality rice nuclear extractions in a medium-throughput manner

When using the chopping procedure, the best results in general were obtained with LB01 nuclear isolation buffer. Every sample triturated by chopping and using LB01 buffer attained low HP-CV values (HP-CV≤5.0%), resulting in preparations of acceptable quality. In contrast, LB01 presented a higher DF average than Otto's buffers when chopping was used, confirming again no correlation between CV and DF in this case. The YF averages obtained when using LB01 buffer and Hanson's buffer were also higher than those yielded using Otto's buffers. The positive results obtained with LB01 buffer may be related to the presence of certain compounds that might counteract the negative effects of some cytosolic compounds such as phenolic substances in suspension (Loureiro et al., 2006b).

Finally, detergents were components of all of the buffers, although the characteristics were not the same. Tween 20 has been confirmed as a weaker detergent, compared to Triton X-100 (Loureiro et al., 2006b). Triton X-100 was used in LB01 and Hanson's isolation buffers at 0.1 and 0.5% (v/v) concentration respectively. However, 0.5% (v/v) Tween 20 was used in Otto's isolation buffers instead. We consider that 0.1% (v/v) Triton X-100 was enough to lyse every pigment extracted and to avoid debris aggregations. Nevertheless, it has been observed that increasing the concentration of detergents such as Triton X-100 and Tween 20 up to 0.5-1% (v/v), in isolation buffers ensures the lysis of chloroplasts in suspension and avoids debris aggregations (Jaroslav et al. 2005). Given the importance of obtaining low debris background, the latest designed nuclear isolation buffers such as General Purpose Buffer (GPB) and Woody Plant Buffer (WPB) include Triton X-100 at 0.5 and 1% (v/v) concentrations respectively (Loureiro et al. 2016), and it would be worthwhile assessing these concentrations could have been assessed.

Bead beating trituration of rice leaf samples produced the best results using Hanson's nuclear isolation buffer. No significant differences were observed between the three assessed isolation buffers in terms of HP-CV, whereas Hanson's and Otto's buffers had lower DF values than LB01. Consequently, there was no correlation between CV and DF observed with bead beating, which was similar to the buffer assessment with chopping. Further, a good YF average was obtained with bead beating using Hanson's buffer. In addition, important cost and time savings were made for sample trituration using Hanson's nuclear buffer with bead beating and the inclusion of β-mercaptoethanol was avoided. We found that bead beating proved to be a suitable procedure that allowed good quality nuclear extracts to be obtained from rice. Moreover, sample analysis was faster than using the traditional chopping procedure, while also decreasing the cost per sample. Therefore, it would be advantageous to implement this trituration procedure when analyzing other plant species, with consideration given to possible variations in trituration time and frequency as well as isolation buffer composition to achieve the best results.

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Chromosome doubling of androgenic haploid plantlets of rice (*Oryza sativa*) using antimitotic compounds

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Chromosome doubling of androgenic haploid plantlets of rice (*Oryza sativa*) using antimitotic compounds

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Abstract

The regeneration of haploid plantlets is considered as a bottleneck in rice anther culture. In this study, an antimitotic chromosome doubling method, simple and efficient, of androgenic haploid plantlets resulted in an efficient doubled haploid obtainment. Through chromosome doubling capacity comparison of the three antimitotic compounds (colchicine, trifluralin and oryzalin), colchicine at 500 and 625 mg/L without supplementing with DMSO was found to be the best antimitotic treatment, with a chromosome doubling capacity of 40%. Furthermore, the in vitro growth of plantlets was followed to analyse the effects of antimitotic compounds. Colchicine treatments were more toxic than dinitroanilines, and colchicine DMSO-supplemented treatments had significant lower values on shoot growth. On the other hand, dinitroaniline compounds impeded root growth, provoked helical growth of shoot and caused the apparition of white nodules in the base of the plantlet due to sprouting abortion. In this study, a protocol for doubled haploid plant recovery was established taking advantage from androgenic haploid plantlets in order to increase the number of doubled haploid plantlets produced after an anther culture protocol.

KEYWORDS

antimitotic, chromosome doubling, colchicine, dinitroanilines, doubled haploid, rice

1 | INTRODUCTION

Doubled haploid (DH) plant lines have duplicated chromosomes inherited from a single donor parental. Complete DH homozygous lines can be obtained through androgenesis, gynogenesis or parthenogenesis within one year, shortening the time in comparison with conventional breeding. DH technology take advantage from a first gametophytic cell at haploid stage, which suffer a spontaneous or induced chromosome duplication during the process to finally obtain a fully regenerated DH plantlet.

Rice DH lines are obtained through androgenesis. Since the first report of anther culture by Niizeki and Oono (1968), many research has been done to overcome limiting factors and optimize the methodology to produce green DH plantlets (Hooghvorst et al., 2018). According to Lentini, Roca, and Martinez (1997), the most important factors affecting anther culture efficiency in rice are as follows: the high genotypic dependency, the low frequency of callus induction and plantlet regeneration, the low percentage of doubled haploids production and the high ratio of albino plantlets. Chromosome duplication in rice is usually spontaneous, around 30%–40% of the regenerated plantlets are spontaneous DHs, but is highly genotype-dependant (Rukmini, Rao, & Rao, 2013).

Doubled haploid technology usually uses antimitotic agents to induce chromosome duplication of haploid cells. Antimitotic agents disrupt plant's cell cycle inhibiting metaphase. In metaphase, α- and β-tubulin dimers are forming the spindle which is essential for polar migration of chromosomes. If the separations of chromosomes are interfered by antimitotic agents, cell results with a doubled haploid chromosome complement (Dhooghe, Laere, Eeckhaut, Leus, & Huylenbroeck, 2011). Colchicine is the most used antimitotic due to its affinity to bind plant tubulins. Nevertheless, colchicine has a high toxicity and side effects in some plant species, such as sterility, chromosome losses or

rearrangements, gene mutation or abnormal growth. Then, other antimitotic agents have been also considered (*e.g.* dinitroanilines). Oryzalin and trifluralin belong to the dinitroaniline class and are antimitotic herbicides that interfere with the normal root development in a number of plant species and have stronger binding affinity to plant tubulins at lower concentrations than colchicine (Ramulu, Verhoeven, & Dijkhuis, 1991).

Antimitotic agents have been barely assayed in rice DH programmes in contrast to other species such as maize (Obert & Barnabás, 2004), pepper (Irikova, Grozeva, & Rodeva, 2011), melon (Dong et al., 2016) or onion (Bohanec, 2002), where antimitotic compound, explant type, time and concentration and exposure treatment have been further optimized. Besides, some authors have successfully treated anthers containing microspores with colchicine in order to boost DH production (Alemanno & Guiderdoni, 1994; Hooghvorst et al., 2018). Furthermore, chromosome doubling of fully regenerated haploid plantlets from anther culture has been demonstrated successful with a colchicine and oryzalin in vitro treatment (Hooghvorst et al., 2018). Therefore, we attempt to report a successful application of different antimitotic agents to improve anther culture process in rice.

The aim of this study is to improve the rice anther culture process by taking advantage of fully regenerated haploid plantlets and treating them with antimitotic agents. Thus, we tested (i) the effect of DMSO combined with colchicine, (ii) the effect of colchicine, oryzalin and trifluralin antimitotic agents at different concentrations, and the (iii) the chromosome doubling ability of colchicine oryzalin and trifluralin at different concentrations in haploid plantlets of rice.

2 | MATERIALS AND METHODS

2.1 | Plant material and haploid obtention

Rice (*Oryza sativa*) haploid plantlets (variety NRVC 980385) obtained from an anther culture protocol was used in our study. Androgenic haploid plantlets were obtained following the protocol of Hooghvorst et al. (2018), using the free-colchicine induction medium 0/0-D1 (N6 standard salts and vitamins fortified with 1 g/L casein hydrolysate, 250 mg/L L-proline, 2 mg/L 2,4 dichlorophenoxyacetic acid, 1 mg/L kinetin, 500 mg/L 2-(N-morpholino) ethane sulphonic acid (MES), 30 g/L sucrose and 3 g/L Gelrite.). Plantlets were maintained in vitro on 17 cm tubes in medium MS (Murashige & Skoog, 1962): 4.4 g/L MS salts & vitamins, 30 g/L sucrose, 0.5 g/L MES monohydrate and 2 g/L gelrite, and pH was adjusted to 5.8. Medium was autoclaved.

2.2 | Chromosome doubling treatments

Regenerated haploid rice plantlets were subcultured in MS medium for two weeks in order to ensure physiological homogeneity between them. Haploid ploidy level of all plants was checked before the experiment to ensure the use of androgenic haploid material. In aseptic conditions, plantlets were trimmed leaving 1 cm of roots and 2 cm of shoots. Trimmed plantlets were introduced on 50 ml tubes filled up with 45 ml of antimitotic treatment solution.

Three antimitotic compounds were used for antimitotic treatments solutions: colchicine ($C_{22}H_{25}NO_6$), oryzalin ($C_{12}H_{18}N_4O_6S$) and trifluralin ($C_{13}H_{16}F_3N_3O_4$). Compounds were dissolved in 1% DMSO or water, depending on the treatment and supplemented with 4 drops/L of Tween 20. For colchicine treatment, two treatments were performed, one supplemented and dissolved in 1% DMSO and one without the DMSO supplement and dissolved in water. Oryzalin and trifluralin treatments were dissolved in 1% DMSO. All antimitotic solutions were filtered by Millipore filters of 0.22 μm (Millipore Corporation, Bedford, USA). Colchicine was assessed at 375, 500 and 625 mg/L, and oryzalin and trifluralin at 8 and 16 mg/L. For each treatment, colchicine-free DMSO, colchicine-supplemented DMSO and dinitroanilines, a control treatment without antimitotic compound was done. A total of 30 plantlets were selected for each antimitotic treatment, and ten plantlets were used for every one of three controls.

The plantlets were incubated with its respective antimitotic treatment for 5 hr on a shaker with orbital agitation at 2.5 Hz. After incubation, plantlets were rinsed with distilled autoclaved water and placed on hormone-free MS medium. All components were supplied by Duchefa (Duchefa Biochemie BV, The Netherlands).

2.3 | Ploidy-level determination

Once plantlets reached sufficient size, samples were collected for flow cytometry ploidy analysis determination following Hooghvorst et al. (2018). The stained nuclei samples were analysed using a Gallios^{TM} Flow Cytometer (Beckman Coulter Inc.,) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centres, University of Barcelona) and a 32-well carrousel. Once ploidy was determined, doubled haploid plantlets were acclimatized in the greenhouse for seed recovery.

2.4 | Statistical analyses

A total of 330 plantlets were treated. In the control treatments, 30 plantlets were treated without antimitotic and divided into Control water (CCW), used to compare with colchicine without DMSO treatment; Control-DMSO (CCD), used to compare with colchicine with DMSO treatment; and Control dinitroanilines (CDN), used to compare with oryzalin and trifluralin treatments. A total of 30 plantlets were treated in each antimitotic treatment. Plantlets treated with colchicine were separated in two groups depending on the concentration of DMSO in the solution (0 and 1%). Antimitotic treatment protocol was performed three independent days with a third of the plantlets each day.

Several parameters were recorded at the third week since the antimitotic treatment: percentage of survival, percentage of sprouting (those plants whose cut first shoot died but plantlet was

TABLE 1 Effect of antimitotic treatment and antimitotic concentration on survival rate (%), sprouting rate (%), plants with helical growth and plants with nodules from aborted sprouts of rice plantlets during the first three weeks after antimitotic treatment and percentage of plants at each ploidy level, haploids (x) and mixoploids with doubled haploid level (x + 2x)

Antimitotic treatment	Antimitotic concentration (mg/L)	no treated plantlets	Survival rate (%)	Sprouting rate (%)	n° plantlets helical growth	n° plantlets with nodules	Haploid plantlets (x) (%)	Mixoploid plantlets with diploid level (x + 2x) (%)
Colchicine DMSO-free	375	30	96.67	16.67*	3	0	64.29	35.70
	500	30	86.67	26.67*	0	0	60.71	39.30
	625	30	93.33	40*	0	0	66.67	33.30
	0 (CCW)	10	100	0	0	0	100	0
Colchicine DMSO-supplemented	375	30	93.33	30*	0	0	68.97	31.00
	500	30	93.33	40*	4	0	57.69	42.30
	625	30	90	60*	2	0	57.14	42.90
	0 (CCD)	10	100	0	0	0	100	0
Trifluralin	8	30	93.33	3.33	6*	10*	100	0
	16	30	86.67	0	9*	11*	92.31	7.70
Oryzalin	8	30	86.67	0	10*	16*	96.15	3.80
	16	30	96.67	0	9*	12*	82.76	17.24
	0 (CDN)	10	100	0	0	0	100	0
Total		330	92.42	0.20	43	49		

Note: Values followed by * in sprouting rate, plants with helical growth and plants with nodules parameters showed significant differences at 5% level in a chi-square test for homogeneity per antimitotic treatment.

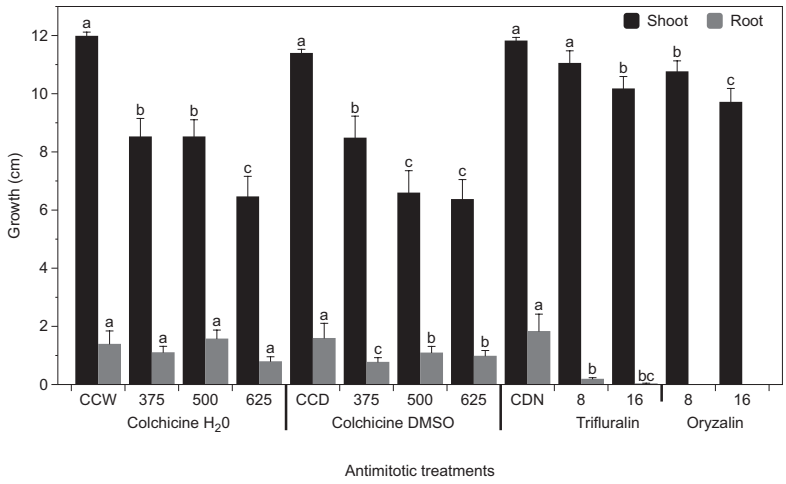

FIGURE 1 Effect on root and shoot growth (cm) after three weeks of antimitotic treatment of cultured in vitro plantlets. Antimitotic treatments were as follows: colchicine solution dissolved in water (Colchicine H_2O) at concentration 0 (control treatment named as CCW), 375, 500 and 625 mg/L; colchicine solution supplemented with DMSO (Colchicine DMSO) at concentration 0 (control treatment named as CCD), 375, 500 and 625 mg/L; and dinitroanilines solution at concentration 0 (control treatment named as CDN), trifluralin and oryzalin, both at 8 and 16 mg/L. Letters denote statistical differences within the corresponding treatment for shoot or root by a Kruskal-Wallis non-parametric test with a Dunn's test for post hoc KW testing ($p < .05$), and vertical bars denote standard error

able to sprout and generate new shoots), number of plantlets with helical growth and number of plantlets with white nodules. Those parameters were analysed with a chi-square test for homogeneity ($p < .05$). Shoot and root growth data from the third week since antimitotic treatment was statistically analysed by a Kruskal-Wallis non-parametric test with a Dunn's test for post hoc KW testing ($p < .05$).

3 | RESULTS

3.1 | Anther culture

A total of 2.361 anthers were sown in 0/0-D1 induction medium as reported by Hooghvorst et al. (2018). Calluses induced from microspores regenerated 824 green plantlets, which 62% were haploids and 38% were spontaneous doubled haploid.

3.2 | Effect of the antimitotic agents

Control treatment supplemented with 1% DMSO (Control CD) and control treatment DMSO-free (Control CW) did not cause plantlet mortality. Nevertheless, two out of three colchicine DMSO-supplemented treatments, 375 and 625 mg/L, resulted in a higher plantlet mortality comparing with same concentration of colchicine DMSO-free (Table 1).

The survival rate after three weeks was maximum at control treatments. All antimitotic treatments had an effect on the survival rate of the plantlets, being always lower when compared with the control (Table 1). Colchicine at 500 mg/L DMSO-free, trifluralin at 16 mg/L and oryzalin at 8 mg/L gave the higher mortality rates for each antimitotic.

The sprouting rate occurred when the first shoot cut died but plantlet was able to sprout and generate new shoots. Then, the sprouting rate recorded was the number of plantlets that overcame the antimitotic stress although being initially strongly affected. Sprouting rate was zero for all of the three controls and 8 and 16 mg/L oryzalin treatments. Furthermore, all colchicine treatments showed significance ($p < .05$) for the main shoot mortality, forcing the plantlet to regenerate new shoots. The dead of the first shoot and the following sprouting rate was highly affected by the concentration of colchicine, the higher the concentration was, the higher was the sprouting rate. (Table 1).

All oryzalin and trifluralin treatments showed significance ($p < .05$) for the rate of helical growing plantlets (from 20% to 40%), although oryzalin and trifluralin treatments showed a significant ($p < .05$) appearance of white nodules in the base of the plantlet after three weeks, from 35% to 61%. Those white nodules changed to black after three weeks.

3.3 | In vitro vegetative growth

The shoot growth of control-treated plantlets (CCW, CCD and CDN) showed significant differences ($p < .05$) (Figure 1). Control treatment CCW (11.99 ± 0.12) and CDN (11.83 ± 0.1) did not show significant differences in the post hoc comparison. Contrary, both

control treatments showed significant differences compared with CCD (11.40 ± 0.13). Root development did not show significant differences (Figure 1).

The shoot growth of colchicine treatments without DMSO showed significant differences ($p < .05$) compared to DMSO-free control (CCW). No significant differences were observed for root growth (Figure 1). Furthermore, all colchicine concentrations showed significant differences when comparing to their control treatment, and 625 mg/L concentration gave the lower value of shoot growth (6.47 ± 0.68). Contrary, DMSO-supplemented colchicine treatments showed significant differences ($p < .05$) for both, shoot and root growth, compared to DMSO-supplemented control (CCD). Finally, colchicine concentration of 625 mg/L gave the lower values for shoot growth and was significantly different when comparing to the 375 mg/L concentration (Figure 1).

By comparison of DMSO-supplemented trifluralin and oryzalin treatments with their DMSO-supplemented control (CDN), significant differences were observed for shoot and root growth ($p < .05$). For shoot growth, all treatment values were lower than the control and significantly different, excepting 8 mg/L of trifluralin (Figure 1). The shoot growth was reduced with higher concentrations of trifluralin (10.18 ± 0.41) and oryzalin (9.72 ± 0.46). Dinitroaniline treatments gave the lower root growth from 0.2 to 0 cm in three weeks (Figure 1). On the one hand, trifluralin treatments lowered root growth from 9 to 43 times comparing with CDN. Plantlets treated with oryzalin (8 and 16 mg/L) did not develop root growth upon three weeks; therefore, no statistical comparison was possible.

3.4 | Chromosome doubling

The ploidy level of the treated plantlets with control treatments and 8 mg/L trifluralin remained haploid. Contrary, the other antimitotic treatment induced a partial chromosome doubling (Table 1). No complete chromosome doubling was reported. Nevertheless, mixoploid plants were considered as chromosome doubled because of their partial doubling. Colchicine treatments resulted in more than 30% of plantlet duplication. Colchicine DMSO-free treatment at 500 mg/L and colchicine DMSO-supplemented at 625 mg/L resulted higher percentages of mixoploid plantlets (x + 2x), 39.30 and 42.90%, respectively (Figure 2). In those two treatments, between 9 and 12 plantlets were chromosome doubled with colchicine from the first 30 haploid plantlets, being colchicine treatment supplemented with DMSO 1%, which resulted in the maximum number of chromosome-doubled plantlets. Trifluralin and oryzalin treatments resulted in less than 18% chromosome doubling, being 16 mg/L oryzalin the best dinitroaniline treatment for chromosome doubling (17.24%).

3.5 | Doubled haploid seed recovery

Chromosome-doubled plantlets were acclimatized in greenhouse conditions and grown until seed set. Self-pollination of panicles resulted in a set of panicles, some with complete filled grains, some with partial sterility and others with complete sterile haploid flowers. All of the mixoploid plants with a doubled haploid ploidy level (x + 2x) produced seed.

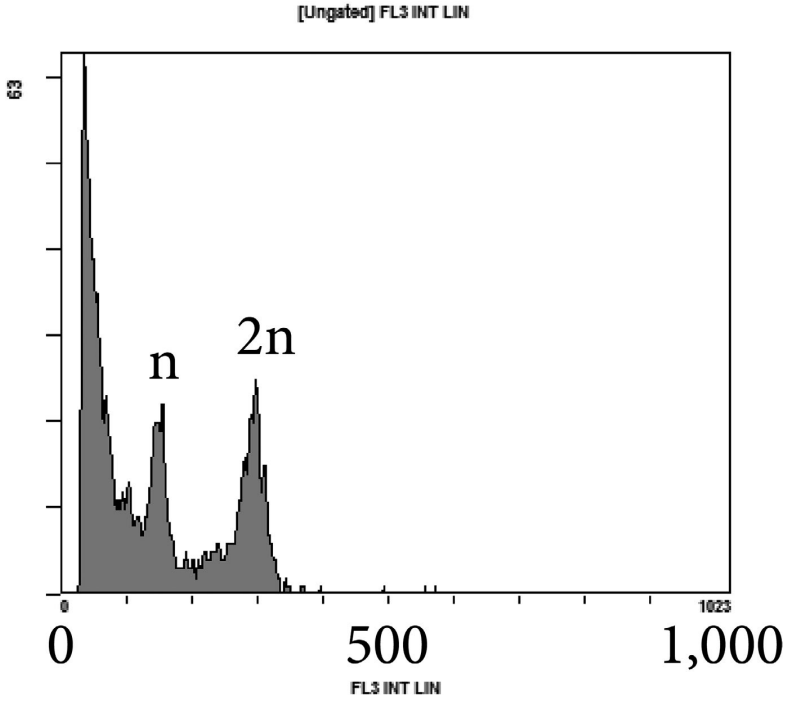

FIGURE 2 Cytometry flow histogram of a mixoploid plantlet (x + 2x) presenting two peaks, at number channel 150 and 300, corresponding to haploid and doubled haploid channels, respectively

4 | DISCUSSION

The obtention of doubled haploids in rice is done through androgenesis applying anther culture. During anther culture, rice microspores usually chromosome double spontaneously allowing the recovery of doubled haploid plantlets. Nevertheless, a ratio of 50%–70% of androgenetic plantlets is a haploid (Hooghvorst et al., 2018;Mishra & Rao, 2016). Therefore, in this study, we present a chromosome doubling methodology of haploid plantlets once they are regenerated to recover them and increase the total efficiency of doubled haploid plants obtained from anther culture procedures in rice.

DMSO is an agent usually used to dissolve the antimitotic compounds. Many authors supplement the antimitotic solution with DMSO because it increments cell permeability and allows an increased absorption of chemicals (Hamill, Smith, & Dodd, 1992). Although its solvent and increased-cell-permeability features in some species DMSO have been declared as a toxic agent that increases the mortality rate of treated plantlets or explants (Dhooghe, Denis, Eeckhaut, Reheul, & Labeke, 2009). DMSO is usually used in a concentration range between 2% and 4% in the antimitotic solutions. In our study, we supplemented less DMSO to the antimitotic solution (i.e. 1%). Control 1% DMSO-supplemented treatment without any antimitotic did not cause any mortality, neither negative effect on root growth. Besides, shoot growth showed a significant reduction due to the presence of DMSO compared to DMSO-free control. These results are not totally in accordance with Dhooghe et al. (2009) or Dhooghe et al. (2011) who reported an increase of mortality when DMSO was applied. The inclusion of a control treatment DMSO-supplemented allowed to observe the negative effect of DMSO on shoot growth in rice plantlets. Contrary, regarding colchicine DMSO-supplemented treatments, a slightly higher mortality was observed in 375 and 625 mg/L when supplemented with DMSO in comparison with same concentrations without DMSO. The mortality of the first shoot was twofold higher when treating plantlets with colchicine DMSO-supplemented; nevertheless, these plantlets overcame the toxicity and were able to sprout. In spite of this reduction, we observed a negative effect of DSMO in shoot growth and in the mortality of the first shoot, forcing the plantlet to sprout. Besides, we did not observe a higher mortality rate in the presence of DMSO and neither a substantial increase of chromosome doubling capacity, as reported previously (Hamill et al., 1992). In summary, colchicine treatments without DMSO performed similarly to DMSO-supplemented ones, a higher shoot growth and a less mortality of the first shoot were reported when dissolving colchicine in water. Then, in rice, DMSO can be considered innocuous and even as a negative supplement because it is the reduction of shoot growth, and therefore, it can be avoided when treating rice haploid plantlets with colchicine for chromosome doubling. Water can be used as colchicine solving agent instead, as pointed by Dhooghe et al. (2011).

Colchicine and dinitroanilines have antimicrotubular activity, and therefore, they act as antimitotic compounds. Colchicine has a strong toxic effect on plant cells; nevertheless, oryzalin and trifluralin are less toxic since they possess higher specific binding affinity to plant tubulins and promote an effective microtubule depolymerization at lower concentrations (Morejohn & Fosket, 1991). In rice, we found that concentrations of dinitroanilines between 8 and 16 mg/L can be considered as high concentrations since mortality ranged from 7% to 14%, being similar or even higher than colchicine concentrations ranging from 375 to 625 mg/L. Hooghvorst et al. (2018) reported a higher mortality rate (34.78%) when rice haploid plantlets were treated using 2.5 mg/L oryzalin. The mortality of the first shoot and the sprouting of secondary shoots of plantlets were observed in all colchicine treatments, which enforces the higher toxicity of colchicine compared to dinitroanilines in plants consideration (Dhooghe et al., 2011). Then, increasing dinitroanilines concentration up to 16 mg/L can be assayed for chromosome doubling in haploid rice plantlets but would eventually increase the plant mortality.

Considering in vitro vegetative growth of treated plantlets, on the one hand, colchicine treatments showed a significant 30 to 50% reduction of the shoot growth, dinitroaniline treatments showed a slightly reduction of shoot growth similar to their control. On the other hand, root growth of colchicine treatments showed similar reduction values as shoot growth (30 to 45%), but dinitroaniline treatments showed root growth reductions from 90% to 100%. Therefore, colchicine treatment caused a reduction of shoot and root growth, and dinitroaniline treatments caused a reduction of shoot and an impediment of root growth. In *Arabidopsis thaliana*, Bao, Kost, and Chua (2001) observed a strong inhibition of root growth and morphogenesis due to the effect of oryzalin treatment, which reduced alfa tubulin expression in roots and caused abnormal cell expansion and defective cytokinesis. Dinitroanilines are used as herbicides and have a severe effect on plant root elongation and development. This effect is due to the depolymerization of microtubules and the stopping of cell division, causing an anisotropic growth and the appearance of square-shaped cells caused by the inhibition of cell division elongation and differentiation (Anthony & Hussey, 1999). In our experiment, we observed a negative effect of dinitroanilines in root growth of rice plantlets. Additionally, all dinitroaniline treatments affected the normal growth of shoots causing left-handed helical growth. This abnormal growth was explained by Thitamadee, Tuchihara, and Hashimoto (2002) as a reduced microtubule stability due to a negative mutation at tubulin intradimer interface of α-tubulins 4 and 6. Antimitotic compounds such as oryzalin caused the negative mutations. Antimitotic compounds inducing left-handed helical growth has never been described in rice. Other abnormalities, such as white nodules or globular structures formation in the plantlet base, were also caused by dinitroanilines compounds. These globular structures were possibly secondary shoots aborted due to the toxicity of the antimitotic.

Then, dinitroanilines are usually described as a less toxic and more efficient antimitotic compounds that could replace colchicine. As reported in this study, trifluralin and oryzalin have a lower chromosome doubling ability than colchicine with the assayed exposure times and concentrations. Taking into account the chromosome doubling dinitroanilines displayed worse than colchicine. In other species, dinitroanilines can induce higher chromosome doubling

efficiencies than colchicine, as reported in haploid onion embryos (Grzebelus & Adamus, 2004) or in *Brassica napus* where trifluralin treatment of microspores provide an efficient chromosome doubling rate. As pointed by Dhooghe et al. (2011), effectiveness of the antimitotic agent is dependent on explants, exposure time and concentration and species and genotypes. Moreover, in spite of the 20–80 times lower concentration of oryzalin and trifluralin than colchicine, the three antimitotic compounds showed a similar mortality rate.

In vitro antimitotic treatment of fully regenerated haploid rice plantlets for chromosome doubling was first applied by Hooghvorst et al. (2018). Few reports have been reported in rice applying antimitotic compounds for chromosome doubling, possibly due to the high spontaneous duplication of rice, which can be around 30%–40% (Mishra & Rao, 2016). Nevertheless, depending on the genotype, doubled haploid plantlet regeneration rate can be low, and a later antimitotic treatment can be helpful to increase the total number of doubled haploid plantlets. Colchicine dissolved in DMSO was the antimitotic treatment with higher doubling chromosome (x + 2x) capacity of 42.30 and 42.90% when treating at 500 and 625 mg/L, respectively. Dinitroaniline treatments gave the worst results of chromosome doubling of the assay. The higher chromosome doubling capacity in rice of colchicine compared with oryzalin was demonstrated by Hooghvorst et al. (2018). Although other authors have reported better doubling capacity of dinitroanilines than colchicine, Greplová, Polzerová, and Domkářová (2009); Grzebelus and Adamus (2004), thus, can be a demonstration that antimitotic compound has higher affinities depending on the specie of use.

5 | CONCLUSIONS

In this study, we have shown the possibility to take advantage of regenerated haploid plantlets from rice anther culture experiments in order to obtain doubled haploid lines using antimitotic treatments. Anther culture in rice has many bottlenecks that difficult the efficiency of doubled haploid plant obtainment. As a two step-protocol, with initial callus development and subsequent green plantlet regeneration from embryogenic callus, anther culture has limiting factors as genotypic dependency, low frequency of callus induction and plant regeneration, high ratio of albino plantlets and low percentage of doubled haploids produced (Hooghvorst et al., 2018; Lentini et al., 1997; Mishra & Rao, 2016). We have analysed: the in vitro development of plantlets after being antimitotic treated to better show the limitations of every treatment; the mortality of plantlets after each treatment to show their toxicity; and the chromosome doubling potential of every treatment to establish the best one. The factors affecting the chromosome doubling of haploid plantlets are as follows: (i) the antimitotic compound used, (ii) the agent used for dissolving the antimitotic compound, (iii) the exposure time and (iv) the condition of the haploid plantlet. We assayed three different antimitotic compounds at different concentrations selected from recommendations of previous works (Dhooghe et al., 2011; Hooghvorst et al., 2018). Our work showed how to increase the percentage of doubled haploids by treating haploid with colchicine at a concentration of 500 and 625 mg/L. Coupling this simple and cheap chromosome doubling protocol to anther culture programs in rice may allow to recover the half of haploid regenerated plantlets and increase the total number of doubled haploid plantlets after a cycle of seed formation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTION

I.H. (corresponding author) contributed in the in vitro work, ploidy-level determination, spikelet phenotype determination and manuscript writing. P.R. contributed in in vitro *work*, ploidy-level determination, data collection and manuscript correction. S.N. contributed in the manuscript correction.

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