Universitat de Lleida

Wheat developmental processes as affected by Ppd and Eps alleles and genetic variation within elite cultivars

Helga Ochagavía Orbegozo

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Universitat de Lleida

TESI DOCTORAL

"Wheat developmental processes as affected by *Ppd* and *Eps* alleles and genetic variation within elite cultivars"

Helga Ochagavía Orbegozo

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Ciència i Tecnologia Agroalimentària

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*To **my parents**,*
because you have given me everything without asking anything,
you have been, are and will be my most immense pride.

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Abstract

Wheat development is critical for adaptation, as it determines time to flowering, and may strongly influence yield determination as it controls duration and rate of initiation/appearance of source and sink organs. Genotypic variation in flowering time is associated with genetic variation of three groups of genes. Two of them regulate the vernalization (*Vrn*) and photoperiod (*Ppd*) sensitivity, whilst the third controls development independent of vernalization and photoperiod determining a sort of earliness *per se* (*Eps*).

The main objectives of the present Thesis was to determine the effects of the introgression of earliness *per se* (*Eps*) and photoperiod (*Ppd*) alleles and the degree of genetic variation among elite current cultivars on the (i) partitioning of the developmental time into vegetative, early and late reproductive phases, (ii) dynamics of leaf and spikelet initiation and (iii) dynamics of leaf appearance and tillering. This is needed as much of the large amount of work done so far has focused mainly on the overall effects on time to flowering.

A number of field studies were carried out to characterize and quantify development of different phases, leaf and spikelet initiation, leaf appearance, tillering, final leaf number (FLN) and spikelets in a set of modern wheat cultivars, and near isogenic lines (NILs) for *Ppd* and *Eps*. In addition the interaction between *Eps* genes and temperature was evaluated under controlled conditions at Lleida and Norwich. In all the experiments dissections were made in order to establish the dynamics of leaf and spikelet initiation and leaf and tiller appearance were recorded periodically.

High variability on flowering time across *Ppd* and *Eps* NILs and set of modern cultivars was found. Photoperiod insensitivity (*Ppd-1a*) alleles conferred different degree of earliness on flowering time depending on the genome in which they were introgressed (A, B or D) the doses of insensitivity as well as of the source of the specific allele. *Eps*–early alleles reduced time to flowering, but again the magnitude of the effect depended on the allele considered as well as on the source or the background for a specific allele**.** Differences in flowering time by *Ppd-1a* were mainly associated to changes on the partitioning of developmental phases, FLN and phyllochron of late leaves. On the other hand, variation in flowering time determined by *Eps* alleles was exclusively due to changes on late reproductive phase. The same result was shown when *Eps* effects were tested under a wide range of temperatures. In the latter study,

the advance of heading was related with an increase in sensitivity to temperature during the late reproductive phase produced by *Eps*–early alleles. Finally, genetic variation on flowering time among modern cultivars was mainly caused by differences on the duration of late reproductive phase and FLN. And this variation was associated with the appearance of leaves after terminal spikelet. A discussion is offered within specific chapters on the consequences of the developmental changes produced by the material studied on the adaptation and yielding capacity of the crop, considering likely trade-offs.

All these findings provide to breeders well characterised resources to optimise wheat development either coarse- or fine-tuning it through different patterns of partitioning of developmental time to flowering considering the effects on organogenesis.

Resumen

El desarrollo del cultivo de trigo es crítico para su adaptación, ya que determina el tiempo a floración y es relevante en la generación del rendimiento, porque controla la duración, la velocidad de iniciación y la aparición de los diferentes órganos que serán fuentes y sumideros. La variación genotípica en la fecha de floración está asociada con la variación genética de tres grupos de genes. Dos de ellos regulan la sensibilidad de la vernalización (*Vrn*) y del fotoperíodo (*Ppd*), mientras que el tercero controla el desarrollo independientemente de la vernalización y del fotoperíodo, determinando un tipo de precocidad *per se* (*Eps*).

El objetivo principal de esta Tesis fue determinar los efectos de la introgresión de los alelos de precocidad *per se* (*Eps*) y de fotoperíodo (*Ppd*), y el grado de variación genética entre los cultivares modernos sobre (i) la partición del desarrollo en las fases vegetativa, reproductiva temprana y tardía, (ii) las dinámicas de iniciación de las hojas y espiguillas y (iii) las dinámicas de aparición de las hojas y ahijamiento. Esto es relevante, ya que gran parte del trabajo realizado hasta ahora se ha centrado principalmente en los efectos generales sobre la fecha de floración.

Se realizaron varios estudios en campo para caracterizar y cuantificar el desarrollo de las diferentes fases, la iniciación de hojas y de espiguillas, la aparición de hojas, el ahijamiento, el número final de hojas (FLN) y de espiguillas en un conjunto de cultivares de trigo modernos, y líneas isogénicas cercanas (NILs) para *Ppd* y *Eps*. Además, se evaluó la interacción entre los genes *Eps* y la temperatura bajo condiciones controladas en Lleida y en Norwich. En todos los experimentos se realizaron disecciones para establecer la dinámica de iniciación de hojas y espiguillas, y se registraron periódicamente la aparición de hojas e hijuelos.

Se encontró una alta variabilidad en el fecha de floración entre las NILs de *Ppd* y *Eps* y en el conjunto de cultivares modernos. Los alelos de insensibilidad al fotoperíodo (*Ppd-1a*) confirieron un grado de precocidad diferente en la fecha de floración dependiendo del genoma en el que se introgresaron (A, B o D), de las dosis de insensibilidad y de la fuente del alelo específico. Los alelos de *Eps*–early redujeron la fecha de floración, pero, la magnitud del efecto dependió del alelo considerado, así como de la fuente o el fondo de un alelo específico. Las diferencias en la fecha de floración causadas por *Ppd-1a* se asociaron principalmente a cambios en la partición de las fases de desarrollo, en el FLN y en el filocrono de hojas tardías. Por otro lado, la variación en la fecha de floración provocada por

alelos *Eps* se debió exclusivamente a cambios en la fase reproductiva tardía. Se mostró el mismo resultado cuando los efectos *Eps* se probaron en un amplio rango de temperaturas donde el adelantamiento de espigazón por los alelos *Eps*–early se relacionó con un aumento en la sensibilidad de la temperatura durante la última fase reproductiva. Finalmente, la variación genética en la fecha de floración entre cultivares modernos fue causada principalmente por diferencias en la duración de la fase reproductiva tardía y en el FLN. Esta variación se asoció con la aparición de hojas después de espiguilla terminal. En los capítulos específicos de la tesis, se ofrece una discusión sobre las consecuencias de los cambios de desarrollo producidos por el material estudiado en la adaptación y en la capacidad de rendimiento del cultivo, teniendo en cuenta las posibles compensaciones.

Los resultados obtenidos en esta Tesis proporcionan a los mejoradores recursos genéticos bien caracterizados para optimizar el desarrollo del trigo, ya sea mediante un ajuste más grueso o más fino a través de diferentes patrones de partición del tiempo de desarrollo hasta la floración, considerando a su vez los efectos sobre la organogénesis.

Resum

El desenvolupament del cultiu del blat és crític per a la seva adaptació, ja que determina la data de floració i és rellevant en la generació del rendiment, ja que controla la durada, la velocitat d'iniciació i l'aparició dels diferents òrgans que seran fonts i embornals. La variació genotípica en la data de floració esta associada amb la variació genètica de tres grups de gens. Dos d'aquests grups de gens regulen la sensibilitat de la vernalització (*Vrn*) i del fotoperíode (*Ppd*), mentre que el tercer controla el desenvolupament independentment de la vernalització i del fotoperíode, i determina un tipus de precocitat *per se* (*Eps*).

L'objectiu principal d'aquesta Tesis fou determinar els efectes de la introgressió dels al·lels de precocitat *per se* (*Eps*) i de fotoperíode (*Ppd*), i el grau de variació genètica entre les varietats modernes: (i) la divisió del desenvolupament en les fases: vegetativa, reproductiva primera i tardana, (ii) les dinàmiques d'iniciació de les fulles i espiguetes i (iii) les dinàmiques d'aparició de les fulles i afillaments. Això és rellevant, ja que la major part del treballS realitzat, fins es centren principalment en els efectes generals sobre la data de floració.

Es van realitzar diferents estudis a camp per caracteritzar i quantificar el desenvolupament de les diferents fases, la iniciació de les fulles i espiguetes, l'aparició de fulles, d'afillaments, el nombre final de fulles (FLN) i d'espiguetes en un conjunt de varietats de blat modernes, i línies isogèniques properes (NILs) per a *Ppd* i *Eps*. A més, es va avaluar la interacció entre els gens *Eps* i la temperatura, en condicions controlades, a Lleida i a Norwich. En tots els experiments es van realitzar disseccions per establir la dinàmica d'inici de fulles i espiguetes, i es va registrar periòdicament l'aparició de fulles i fillols.

Es va trobar una alta variabilitat en la data de floració entre les NILs de *Ppd* i *Eps* i en el conjunt de varietats modernes. Els al·lels d'insensibilitat al fotoperíode (*Ppd-1a*) van conferir un grau de precocitat diferent en la data de floració depenent del genoma en el que es van introgresar (A, B o D), de les dosis d´ insensibilitat i de la font de l'al·lel específic. Les diferències en la data de floració causades per *Ppd-1a* es van associar principalment a canvis en la divisió de les fases de desenvolupament, en el FLN i en el "phyllochron" de fulles tardanes. D'altra banda, la variació en la data de floració provocada per al·lels *Eps* va ser causat exclusivament a canvis en la fase reproductiva tardana. Es va veure el mateix resultat quan els efectes *Eps* es van provar en un ampli rang de temperatures on l'avançament de l'espigueig per els al·lels *Eps–early* es va relacionar amb un augment en la sensibilitat de la temperatura durant la darrera fase reproductiva. Finalment, la variació genètica en la data de

floració entre varietats modernes fou causada principalment per diferències en la durada de la fase reproductiva tardana i en el FLN. Aquesta variació es va associar amb l'aparició de fulles després de l'espigueta terminal. En els capítols específics de la tesis, s'ofereix una discussió sobre les conseqüències dels canvis en el desenvolupament produïts pel material estudiat en l'adaptació i en la capacitat de rendiment del cultiu, tenint en compte les possibles compensacions.

Els resultats obtinguts en aquesta Tesis proporcionen als milloradors recursos genètics ben caracteritzats per optimitzar el desenvolupament del blat, ja sigui mitjançant un ajust més o menus fi a través de diferents patrons de divisió del temps de desenvolupament fins la floració, considerant els efectes sobre l'organogenesis.

Chapter I

General Introduction

1. Chapter I: General Introduction

1.1. Context: future global demand for wheat

Wheat is the most widely grown crop because of the intrinsic value of the wheat flour but also because it is well adapted to a large range of temperatures, daylengths, water regimes and nutritional levels. It is, therefore, grown at almost all latitudes and altitudes where arable land is available. Therefore, wheat plays a major role on global food security, providing 20% of the calories and proteins to the world's population (Braun *et al*., 2010).

Wheat yield has increased linearly at a constantly high rate since the onset of the Green Revolution to the late 1980's and early 1990's, but when taking into account the last decades the trend is actually more hyperbolic in nature and can be better fitted by a bi-linear than by a linear regression (Fig. 1). This means that gains in yield have been worryingly diminishing when demand did not stop growing. Currently, wheat yields are growing at an average rate of less than 1% (Ray *et al.*, 2013 and Fig. 1 inset) and, in fact, important regions are actually exhibiting yield stagnations (Brisson *et al.*, 2010; Mackay *et al.*, 2011; Ray *et al.*, 2012), as warned several years before (Calderini and Slafer, 1998). If this rate is not substantially increased (see difference between expected [solid line] and required [dashed line] trends in yield in Fig. 1.1) we risk to "go back in time" increasing again malnourishment (Bruinsma, 2009). And the scenario is even more complex now: we started to use grains, that before were only used for food, to produce biofuels (Fischer *et al.*, 2009), and the demand is increasing beyond the increase in population as there is also an increased demand *per capita* due to shifting diets (Reynolds *et al.*, 2016). And finishing with can be seen as a "perfect storm", impacts of climate change, i.e. increased temperatures and water deficits, raise the risks of decreasing yield (Porter and Semenov, 2005). For instance, it has been estimated with simulation models that wheat yields may decline by 6% per each 1ºC increase in mean temperature (Asseng *et al.*, 2015).

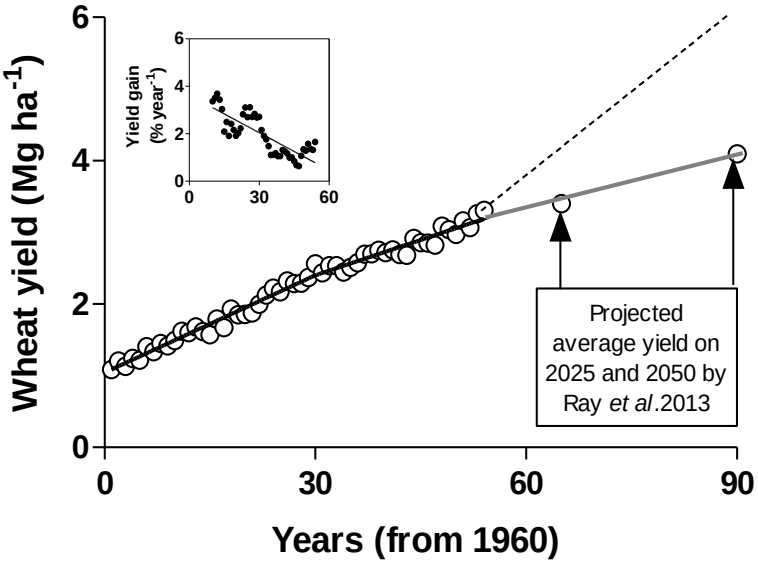

Fig. 1.1. World average wheat yield from 1961 to 2014 and two values of projected average wheat production taken from Ray *et al*. (2013). Inset is the calculated relative gains in yield per decade finishing in each year from 1970 to 2014. Data of world average wheat yield were obtained from FAO (http://www.fao.org/faostat/en/#data/QC). Solid line was fitted data from 1961 to 2014 with a bi-linear (main panel; R^2=0.986, *P<0.001*) or linear (inset; R^2=0.653, *P<0.001*) regression, grey line is the projection of the current trend (should the future increase maintain current rates), and dashed line represents the trend in yield that would be required to match the increase in demand by 2050 (see text).

In this complex context, it has been estimated that wheat production should be increased by, at least, 50% by 2050 (Rosegrant *et al.*, 2009; Ray *et al.*, 2013; Fischer *et al.*, 2014).

The routes to achieve an increase of wheat production could hardly include a significant expansion of the growing area without causing negative effects on long-term sustainability (Reynolds *et al.*, 2012). Thus, the only sustainable solution to increase wheat production is through increasing crop yields on currently exploited agricultural soils (Hall and Richards, 2013) with the selection of cultivars with genetic variability that develop high-yielding (Albajes *et al.*, 2013). But, regretfully, genetic gains in yield -that were very large in the few decades following the Green Revolution- are currently also much smaller (e.g. Sadras and Lawson, 2011; Lopes *et al*., 2012) and in some regions they have been actually negligible in recent decades (e.g. Acreche *et al.*, 2008; Graybosch and Peterson, 2010; Matus *et al.*, 2012). Therefore, it is necessary to promptly recover higher rates of genetic gains in yield.

To increase the likelihood of increasing the rates of genetic gains in breeding it would be convenient to identify physiological attributes that determine yield (simply because the more we understand something the less difficult it would be to manipulate it), and genetic factors

controlling them. Among the different processes controlling yield in wheat (and any other field crop), developmental processes are critical (e.g. Slafer *et al.*, 2015). Therefore, in this Thesis, the work is focused to understand developmental attributes of the crop responsible for adaptation, and generation of organs that will become sources and sinks for yield determination.

1.2 Crop development and adaptation

Crop adaptation consists of adjusting crop development phases to the most favourable conditions, in particular, the timing of flowering through best combinations of sowing dates and cultivar phenology (Zheng *et al*., 2015). Therefore, wheat adaptation is mainly due to the variation of flowering time. If flowering time is too early, cold temperatures could cause male sterility whilst when flowering time is too late, the period of grain number determination is exposed to lower photothermal quotients (Fischer, 1985) and grain growth will be more likely exposed to high temperatures causing heat stress (Slafer *et al*., 2015). Furthermore, crop development may also improve cultivar adaptation by reducing the risk of biotic stresses in some growing regions. Early-maturing was related with a lower rust damage in wheat (Park *et al*., 2009) and with a decrease stem rot damage (Ploper, 2004).

In addition, when considering no only time to anthesis but also (i) partitioning of that developmental time into different phases, and (ii) the dynamics of generation of leaves and spikelets, crop-developmental attributes might also be useful to identify opportunities for modifying developmental patterns, which in turn may affect yield, beyond the effects of adaptation (e.g. Slafer *et al.*, 2005; Miralles and Slafer, 2007).

1.2.1. Flowering time (anthesis)

Flowering time involves phenological events controlled by the genetic background and environmental factors, which determines changes in the external morphology of the plant (macroscopic changes) and apex development (internal and microscopic changes) (Fig.2.2). Time to flowering may be understood as the consequence of either (i) the duration of the different developmental phases: vegetative, early-reproductive and late-reproductive phases (e.g. Slafer and Rawson, 1994; Kirby *et al.*, 1999); or (ii) the number of leaves initiated in the main shoot (during the vegetative phase) and phyllochron, the time elapsed between the appearance of successive leaves (Jamieson *et al.*, 1998). Both approaches are actually more complementary than conflicting.

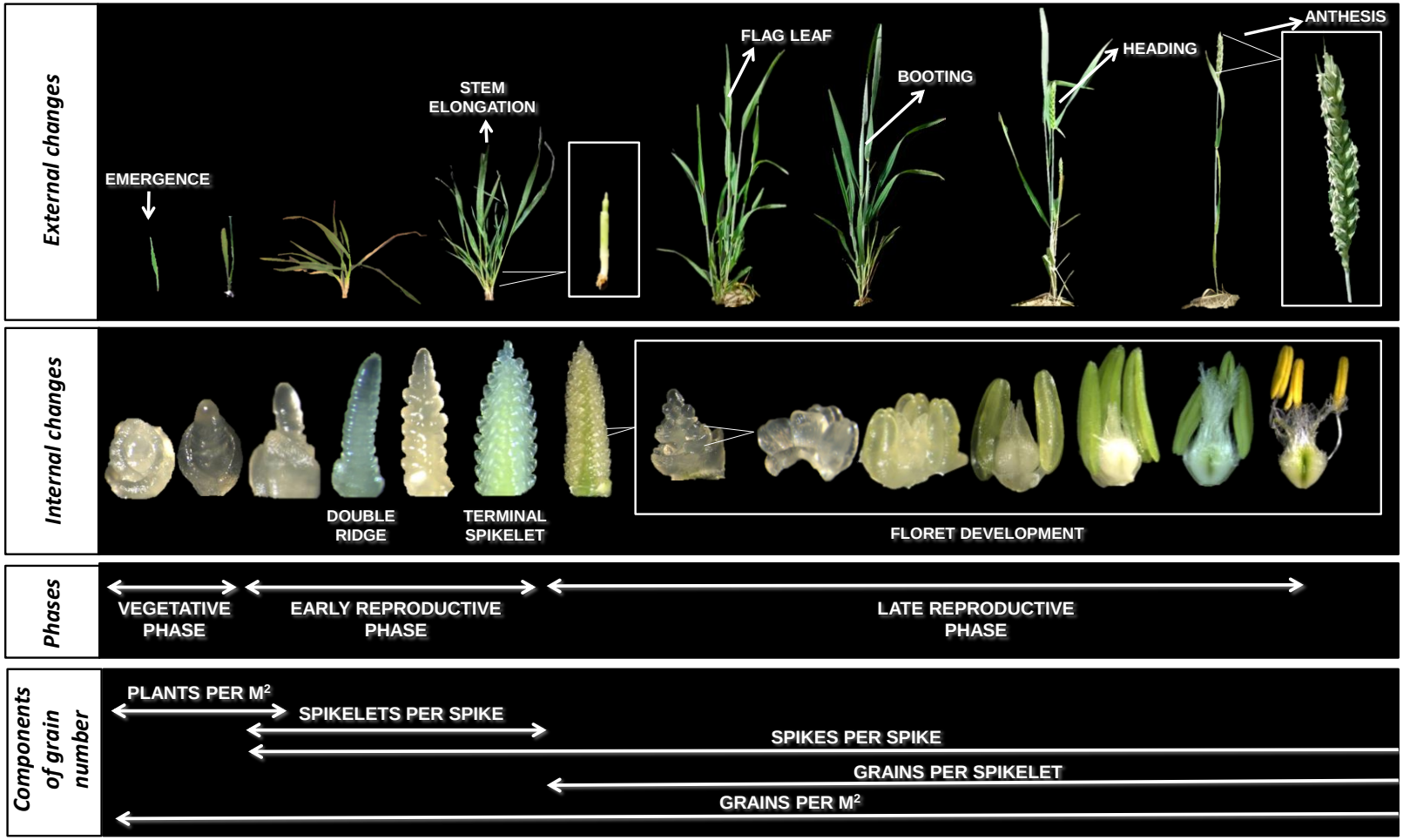

Fig. 1.2. Scheme of growth and development wheat. In the top box, the macroscopic changes: emergence, stem elongation, flag leaf, booting, heading and anthesis are presented. In second box, the microscopic changes in the apex are shown. Third box represents the three developmental phases in which this Thesis is focused. The bottom box presents the timing when the components of grain number per unit land area are generated within and across these phases.

1.2.2. Developmental phases

As illustrated in Fig. 1.2, time to flowering is composed of different developmental phases in which different organs (which will be later sources and sinks determining yield) are being formed (Slafer and Rawson, 1994). These phases are delimited by changes that are produced in the apex. These are:

(i) Vegetative phase, from seedling emergence to floral initiation– leaf primordia are initiated.

(ii) Early reproductive phase, from floral initiation to terminal spikelet –all spikelets are formed. Terminal spikelet is the stage when no more meristematic tissue is left on the very juvenile spike and therefore when the maximum number of spikelets is established.

(iii) Late reproductive phase, the period between terminal spikelet and anthesis. In this period stem internodes elongates while florets develop within the spikelets, firstly all florets are initiated and then most of them go through a mortality phase. Maximum number of floret primordia is firstly produced and then the number of fertile florets is reached at anthesis.

As yield is most frequently limited by sink-strength during grain filling (e.g. Borrás *et al.*, 2004) grain number is the critical component determining response of yield to genetic and management manipulations (Slafer *et al.*, 2014). The number of grains set by the crop is the consequence of sub-components that are being produced throughout the crop cycle from sowing to anthesis (Slafer and Rawson, 1994 and Fig. 1.2). Therefore, this Thesis is focused on the three phases (vegetative, early reproductive and late reproductive) when regarding the phasic development of the crop. Although during grain filling crop yield is sink-limited, during the pre-anthesis development the generation of grains per m^2 is source-limited, as manipulations of resources (radiation, nutrients, water) and signals (photoperiod, temperature) before anthesis produce parallel responses in grain number and yield (e.g. Fischer, 2011; González *et al.*, 2011; Ferrante *et al.*, 2013). Therefore, the Thesis is focused on the differentiation and appearance of vegetative organs (leaves and tillers) as well as on the differentiation of spikelets, the reproductive inflorescence where grains are located when regarding the development of crop organs (Fig. 1.2).

1.2.3. Leaf appearance and phyllochron and their relationship with apex development

After sowing, seed imbibition takes place and initiation of leaf primordia is recovered (leaf initiation started in the mother plant during seed filling in the previous generation producing embryos with c. 4 leaf primordia; Hay and Kirby, 1991). Leaf initiation continues until the onset of floral initiation. The dynamics of leaf initiation is strongly linear with time (at constant temperature or with thermal time in field conditions; Kirby, 1990) and the reciprocal of the rate of leaf initiation (i.e. the period of time or thermal time between the initiation of two consecutive leaf primordia) is the leaf plastochron. The duration of the period from sowing to floral initiation and plastochron (together with the number of primordia developed in the embryo) determine the number of leaves that the main shoot will have, which in turn is one of the determinants of the duration to flowering time, together with the rate of leaf appearance (the reciprocal of this rate is known as phyllochron). When the number of leaves initiated exceeds a certain threshold (e.g. 8), there are two different rates of leaf appearance, one for early leaves and another for late leaves. Generally, the phyllochron of early leaves is shorter (and the rate of leaf appearance faster) than that of late leaves (Miralles and Slafer, 1999). The onset of tillering occurs approximately three phyllochrons after seedling emergence. Dynamics of tillering can be fitted by a tetralinear regression where tillering takes place until resources become a limiting-factor reaching a maximum number of tillers and then the mortality of tillers starts until the number of fertile tillers per plant (or per m^2) is reached, close to anthesis. Tiller emergence is related with leaf appearance (Hay and Kirby, 1991). After floral initiation, the apex differentiates spikelet primordia (the reciprocal of the rate of spikelet initiation is its plastochron). Traditionally, double ridge was used to indicate the transition from vegetative to reproductive apex, because this stage is the first morphological (albeit microscopic) indication that the apex is unquestionably differentiating spikelets. However, spikelet primordia initiation normally starts well before double ridge (as single ridges, morphologically indistinguishable from leaf primordia), without any evident micro-morphological indication. Therefore, in order to date the timing of floral initiation it is necessary to analyse the relationship between accumulated number of primordia and thermal time, and inserting in that relationship the final leaf number and then estimating when the following primordia was initiated (Miralles and Slafer, 1999). Spikelet initiation finishes with the terminal spikelet stage, when the maximum number of spikelets per spike is established.

Wheat development is controlled by environmental and genetic factors. Understanding the genetic factors controlling the dynamics of developmental processes, as well as recognizing

the degree of variation in the determinants of these dynamics among elite material, is critical for the use of this knowledge in breeding (Slafer *et al.*, 2014).

1.3. Genetic control of wheat development

Bread wheat is hexaploid with a genome size estimated at ~17 Gbp, composed of three genomes (A, B and D) which are results of the hybridisation events. Bread wheat presents more complex inheritance than diploid wheat due to the presence of several loci involved in the same trait (Slafer *et al.*, 2015).

Wheat development is mainly controlled by the allelic combination of three genes that regulate sensitivities to both photoperiod (*Ppd*) and vernalisation (*Vrn*), and genotypic differences in rate of development independent of photoperiod and vernalisation (*Eps*). The wide wheat adaptation depends of large allelic variation of these genes (Snape *et al.*, 2001).

Understanding the genetic control of wheat development is useful for plant breeders to fine-tuning the duration of development to a particular environment. Regarding the identification of the effects of particular genes, this Thesis is focused on the effects of *Ppd* and *Eps* genes.

1.3.1. Photoperiod genes (Ppd)

Wheat is a "long day plant"; it means that it flowers earlier when exposed to relatively long days than when growing under short days, as sensitivity to photoperiod in wheat is quantitative (Slafer *et al.*, 2015). Developmental responses to photoperiod are more complex than the universal response to temperature because, variation in sensitivity between genotypes includes insensitivity, and sensitivity varies with developmental stage. Daylength is perceived by leaves and the stimulus is passed to the apex. Many species present a juvenile phase after emergence that is insensitive to photoperiod warranting a minimum duration of the vegetative phase and a minimum leaf number. In wheat this juvenile phase has not been reported (Slafer and Rawson, 1995), therefore the early perception of the photoperiod takes place immediately after seedling emergence. *Photoperiod-1 (Ppd-1,* also named as *PRR37)* genes are the main genes that control response to photoperiod in wheat (*Ppd-D1*, *Ppd-B1* and *Ppd-A1*). They are located on the short arms of chromosomes of group 2 (Beales *et al.*, 2007). Alleles conferring photoperiod insensitivity are categorised with *a* suffix, whereas wild type alleles (which confer sensitivity to photoperiod) are designated with a *b* suffix (McIntosh *et al*., 2003).

There have been studies considering the effect of *Ppd-1* alleles, but they mainly analysed the strength of the effect of the different *Ppd-1* alleles (Snape *et al.*, 2001; Beales *et al.*, 2007; Jones *et al.*, 2016), the effect of the doses of these alleles (Shaw *et al.*, 2012) or eventually the effect of the source of a particular allele (Bentley *et al.*, 2011; Díaz *et al.*, 2012) on time to heading or anthesis almost always under controlled conditions. However, as far as I am aware, it has been never analysed the combined effect of genomes, doses and sources, even less their effects on more detailed developmental processes than time to anthesis. Therefore, in the present thesis, I focused on this, almost always under field conditions. Previous results, indicated that the stronger effect on photoperiod insensitivity is associated to *Ppd-D1a* allele (Snape *et al.*, 2001), which has a 2kb deletion upstream of the coding region causing a misexpression of the gene and an induction of floral activator *FT1* in short days (Beales *et al.*, 2007). Worland and Sayers (1996) showed that photoperiod insensitivity in European wheat varieties was due to introduction of *Ppd-D1a* allele into them, presenting advantages on yield of over 35% and 15 % in Southern and Central European environments, respectively. Although *Ppd-D1a* is generally considered to have a stronger effect than the other two *Ppd-1a* alleles (Worland, 1999), there are some inconsistencies in the literature. Law *et al.* (1978) found that *Ppd-B1a* presented the most potent effect followed of *Ppd-A1a* and *Ppd-D1a*. Tanio and Kato (2007) showed that *Ppd-D1a* and *Ppd-B1a* alleles had a similar strong effect on insensitivity to photoperiod. Díaz *et al.* (2012) reported that *Ppd-B1a* caused also an advancement of flowering time. On the other hand, *Ppd-A1a* alleles (that predominate in durum wheat; Bentley *et al.*, 2011) reduced flowering time producing an effect on photoperiod insensitivity intermediate between *Ppd-D1a* and *Ppd-B1a* (Bentley *et al.*, 2011). Generally, lines with photoperiod insensitivity on two or three genomes increased the earliness on flowering time respect to lines with only one dose of *Ppd-1a* alleles (Shaw *et al.*, 2012).

1.3.2. Earliness per se genes (Eps)

Eps genes are responsible for variation of flowering time when photoperiod and vernalisation requirements are satisfied (Appendino and Slafer, 2003). The effect of these genes are much less understood than those of *Ppd* and *Vrn* genes, and they are generally identified as QTL (Snape *et al.*, 2001). One reason why the knowledge about *Eps* genes is scarce could be that they have been often mapped in crosses segregating for *Ppd* and *Vrn* genes and their effects could mask those of *Eps* genes (Zikhali *et al.*, 2015).

In *Triticum monococcum*, Bullrich *et al.* (2002) mapped a QTL for an *Eps* locus located in a

distal region of chromosome 1A^{m}L (*Eps-A^{m}1*). Heading time was more affected by this gene on controlled environmental condition than on natural conditions. Later, Lewis *et al*. (2008) found that the late allele, *Eps-A^{m}1-l*, caused longer vegetative and early reproductive phases inducing a higher spikelet number per spike. *Molybdenum Transporter 1 (MOT1)* and *Filamentation Temperature Sensitive H (FtsH4)* have been proposed as candidate genes for *Eps-A^{m}1*. Both of genes are expressed in apexes, as it is expected for *Eps-A^{m}1* candidates (Faricelli *et al.*, 2010). There are a large number of *Eps* QTLs: for instance, Griffiths *et al.* (2009) located *Eps* genes on chromosomes 1B, 1D, 2A, 3A, 3B, 4B, 4D, 5A, 5B, 6A, 6B, 7A, 7B and 7D on four doubled haploid (DH) mapping populations (Spark x Rialto, Charger x Badger, Avalon x Cadenza and Rialto x Savannah), which represent a diversity in European winter wheat germplasm. From this study, *Eps-D1* was identified on the long arm of wheat chromosome 1 (1DL), a likely orthologue of *Eps-A^{m}1*. Zikhali *et al*.(2014) validated a 1DL QTL as *Eps* effect under both field and controlled environments conditions using four independent pairs of NILs developed from cross between Spark and Rialto.

Understanding the effects of *Eps* genes will facilitate the fine-tuning of flowering time in breeding programs. In this Thesis, I analysed the effects of *Eps* genes on components of time to anthesis as well as on the generation and appearance of vegetative and reproductive organs, though determining the differences between a large number of NILs for *Eps* genes on chromosome 1D (considering two independent sources of the allele) and 3A.

Temperature is the only environmental factor that affects to all the periods of development; therefore all the developmental phases and all genotypes are sensitive to temperature (Miralles and Slafer, 1999). High temperatures accelerate the rates of development, reducing the duration of the phases (Slafer and Rawson, 1994). As the response of development to temperature is universal, it allows expressing the duration of phases in thermal time (Monteith, 1984). This model assumes a linear relationship between temperature and rate of development within the cardinal thresholds of base and optimum temperatures. Both of them can be vary between varieties (Slafer and Rawson, 1994) and during life cycle (Slafer and Savin, 1991). Perhaps due to the universal nature of temperature effects on developmental process, variation in (and genetic bases for) sensitivity to temperature has not been investigated as much as for photoperiod and vernalisation. Furthermore, *Eps* genes were proposed to be thermo-sensitive (Slafer, 1996), which would imply they would interact with temperature (Bullrich *et al.*, 2002), and would be the genetic bases for different sensitivity to this factor. Previous work testing the hypothesis that *Eps* genes interact with temperature considered only two different thermal regimes and mainly time to anthesis. In this thesis, I

compared several developmental processes under a wide range of temperatures in *Eps*-NILs.

1.4. Genetic variation among elite wheats

Determining the degree of genetic variation among elite wheats in traits responsible of increasing wheat yield is important to develop prospective crosses (Slafer, 2003). It is true that breeding can achieve satisfactory results from wide crossing strategies exploiting available biodiversity within and between species. But this is only preferred when the trait objective of improvement is relatively simple and controlled by major genes. On the other hand, when aiming to increase complex traits (e.g. partitioning of developmental time, rates of organ differentiation and so on), breeders are reluctant to use any approach other than 'pyramiding' traits over their best existing materials; restricting crosses to elite material (Rattey *et al*., 2009).

Within the elite wheats those unquestionably preferred are modern and high-yielding wheats that present an optimum flowering time in the targeted regions, because flowering time is critical to crop adaptation (Slafer *et al.*, 2015). Then, optimizing the duration of developmental phases to time to anthesis may allow to increase the components of grain number produced during this stages (Slafer *et al.*, 2001). However, little is known about genetic variation among modern cultivars in partitioning of developmental time into different phases, in dynamics of leaf and tiller appearance and in leaf and spikelet initiation rates. Therefore, in the research reported in this Thesis, I quantified genetic variation among modern cultivars in all these traits.

1.5. Objectives and hypothesis

The major objective of this Thesis is to determine changes in

- flowering time (which is essential to adaptation)
- partitioning of the developmental time into vegetative, early and late reproductive phases
- leaf- and spikelet-plastochron
- dynamics of leaf appearance and tillering

due to

- genetic variation among elite germplasm formed by current commercial cultivars

- the introgression of earliness *per se* (*Eps*) and photoperiod (*Ppd*) alleles.

The general hypothesis was that not only time to flowering but also duration of each of the sub-phases, the dynamics of leaf and spikelet initiation and phyllochron, tiller appearance and tiller mortality would

- exhibit genetic variation, even when restricting the germplasm to modern commercial cultivars well adapted to the region
- be reduced (duration of phases) and will not be affected (primordia initiation rates) or only slightly reduced (phyllochron, rates of tillering and maximum number of tillers) by the introgression of *Ppd* insensitive alleles (*Ppd-1a*) or *Eps* -early alleles.
- Consequently there will be genotypic differences between modern cultivars as well as effects of *Ppd-1a* and *Eps*-early alleles on the coordination between leaf+spikelet initiation and leaf emergence, and between tillering and leaf emergence dynamics.

Consequences of this hypothesis is that (i) the duration of all sub-phases of time to anthesis would be similarly responsible for differences in the latter, (ii) whenever the duration of a sub-phase were reduced, this would concomitantly imply a parallel reduction in the number of primordia initiated in that phase, and (iii) that FLN would be more relevant than phyllochron in determining differences in or responses of time to anthesis.
Furthermore, there was an additional aim of determining and quantifying the interaction between *Eps* and temperature on all the traits mentioned above under a wide range of growing temperatures, ascertaining whether the *Eps* alleles may affect the threshold temperatures of developmental processes in wheat.

1.6. Outline of this Thesis

This Thesis contains eight chapters. They include this introductory chapter (**Chapter I**), followed by a description of the general procedures and the methodology that was used in most experiments (**Chapter II**) reported in each of the five experimental chapters (**Chapters III, IV, V, VI, VII**) and a final chapter of general discussion and conclusions (**Chapter VIII**).
Each experimental chapter is based on papers which have been published, submitted or prepared to be submitted to international journals. This approach has the advantage that each chapter can be read independently of the others, but the counterpart is that there are unavoidable repetitions, especially in their introduction and material and methods sections.

For this reason, the main body of this introductory chapter may be seen as less "encyclopaedic" as usual and only dealt with the general aspects that are behind the proposed aims and hypotheses (more specific aspects were left in the introduction section of each individual experimental chapter).

Chapter III and Chapter IV determined the changes on flowering time and its components and consequently, in the formation of the organs, caused by photoperiod insensitive alleles combining different alleles, doses and homoeoalleles.

Chapter V evaluated variation on flowering time and its components and the organs formed during early phases induced by *earliness per se* alleles combining different locations of QTL, parents and backgrounds of near isogenic lines (NILs).

Chapter VI explored the interaction between *earliness per se* alleles and temperature through a wide range of temperatures and estimations of base temperature as affected by *Eps* alleles were reported.

Chapter VII presented the degree of genetic variation in many developmental processes of various degrees of complexity within elite material such as modern well adapted cultivars.

Finally, Chapter VIII offers a General Discussion in which it is briefly summarised the context, made explicit the different degrees of originality of the research, recapitulated key results across experimental chapters and highlighted the main contributions to knowledge.

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Chapter II

Materials and methods

2. Chapter II: Materials and methods

2.1. General conditions and treatments

Eight experiments were carried out from 2012/13 to 2015/16 growing seasons. Experiments I to VII were carried out in North East of Spain (province of Lleida, Catalonia) under field conditions in plots, except experiment VIII where genotypes were grown in pots under controlled conditions at different temperature regimens in growth chambers at the John Innes Centre (Norwich, UK) and at the ETSEA Campus (Lleida, Spain).

- ✓ **Experiments I and II** were performed during 2012/13 and 2013/14 growing seasons nearby Bell-lloc d´Urgell (41.63°N, 0.78°E). In both experiments the soil was classified as a complex of *Calcisol petric* and *Calcisol haplic*, following the soil classification of FAO (1990). Experiments were sown on optimum dates for Mediterranean region of Spain at a rate of 300 seeds m^{-2} and 350 seeds m^{-2} respectively. Treatments were nine modern cultivars (*Triticum aestivum L*.). Eight of them (Atae, Arthur Nick, Califa Sur, Garcia, Ingenio, Nogal, Sensas and Rodolfo) are commercial cultivars well adapted to this region (GENVCE) and the other one breeding line was experimental, but during the experiments was registered as a new cultivar with the name of Tribat I33. Each experimental plot consisted of 1. 2 m x 4 m (4.8 m^2). Treatments were arranged in a randomised complete block design with three replications.
- ✓ **Experiments III and IV** were performed in 2012/13 and 2013/14 growing seasons close to Bell-lloc d´Urgell (41.63°N, 0.78°E), in the same experimental field than Exps. I and II. Experiments were sown on 24 November 2012 and 12 November 2013 at a rate of 300 seeds m^{-2}. Treatments consisted of 12 near isogenic lines (NILs) for *Ppd-1* alleles of hexaploid wheat developed at the John Innes Centre (Norwich, UK). The 13 experimental genotypes were the "wild type" Paragon (a wheat cultivar with a strong sensitivity to photoperiod but insensitive to vernalisation) and 12 NILs in which *Ppd-1a* alleles were introgressed on chromosome 2 of the A, B and/or D genomes of the wild type. Treatments were arranged in a complete randomised design with different number of replications (ranging from one to five replications, depending on availability of seeds) in 2012/13 and in a randomised complete block design with three replications in 2013/14 (when we had no seed limitation due to the

multiplication done in the previous season). In all cases experimental units (field plots) were 1.2 m width (6 rows 0.20 m apart) and 4 m long.

- ✓ **Experiments V, VI and VII** were carried out during 2012/13, 2013/14 and 2014/15 growing seasons at Algerri, 41.8ºN, 0.64ºE (in 2012/13 and 2013/14 growing seasons) and at Bell-lloc d´Urgell, 41.6ºN, 0.78ºE (in 2014/15 growing season). Soil types were classified as *Fluvisol calcari* and *Calcisol petric* (in 2012/13 and 2013/14 growing seasons) and as a complex of *Calcisol petric* and *Calcisol haplic* (in 2014/15 growing season) according to the soil classification of FAO (1990). Sowing dates were on 2 November 2012, 7 November 2013 and 14 November 2014 at a rate of 300 seed m^{-2}. Treatments consisted of thirty-two near isogenic lines (NILs) segregating for *Eps* genes. Near isogenic lines were developed at John Innes Centre (Norwich. UK). Twenty-four NILs came from the cross Avalon x Cadenza, twelve of them had *Eps* effect on chromosome 1DL (6 lines with *Eps*- early and 6 lines with *Eps*- late), whilst the other twelve presented the QTL on 3AL (6 lines with *Eps*- early and 6 lines with *Eps*- late). Finally, eight NILs were made from cross Spark x Rialto with *Eps* effect on 1DL (4 lines with *Eps*- early and 4 lines with *Eps*- late). Each NILs pairs have a NIL homozygous at the QTL for the Avalon/Spark allele and another NIL homozygous at the QTL for the Cadenza/Rialto allele. For NILs with *Eps* effects on 1DL, Cadenza and Spark had a deletion where a lot of loci were lost whose predicted function was an earliness flowering time, whilst for some NILs with *Eps* effects on 3AL was Avalon who provides an advancement on flowering time. NILs with *Eps* effect on 1D (both those that came from cross Avalon x Cadenza and Spark x Rialto) were sown in 2012/13 and 2013/14 growing seasons whilst NILs with *Eps* effect on 3A from Avalon x Cadenza were sown in 2013/14 and 2014/15.

  Treatments were arranged in a completely randomised design with eighteen (for NILs from AxC) and twelve (NILs coming from SxR) replications, as each line had three replicates. Plots were 5 m long with six rows 0.20 m apart (in 2012/13 and 2013/14 growing seasons) and 4 m long with six rows 0.20 m apart (in 2014/15 growing season).
- ✓ **Experiment VIII** was performed in the facilities of University of Lleida (UdL) and of John Innes Centre (JIC) using growth chambers (GER-1400 ESP, Radiber, S.A and CER, respectively). Before starting the experiments in the growth chambers, all seedlings were vernalised in order to minimise any interference from vernalisation

responsiveness. For this purpose, pots (200 or 400 cm^3, at UdL and JIC, respectively) were filled with a mixture of 30% peat and 70% soil (UdL) and cereal mix compost composed of 40% peat and 40% soil and 20% grit (JIC) and seeds were sown at a rate of a single seed per pot. A 40-50% of extra pots were sown to choose at the beginning of the experiments the uniforms plants and reducing the experimental error due to plant-to-plant variations. After sowing and irrigation, pots were left at room temperature (*c*. 20 °C) during 1 day for the germination process to be triggered, the metabolic activity fully recovered (and vernalisation stimulus perceived). The day after sowing, pots were located in a cool room at 4ºC in dark during 49 days. After 49 days, all the selected pots were transferred to the corresponding growth chamber. Chambers were always set under long day conditions (18 h) and subjected to light intensity of 110 µmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) at plant level in UdL chambers, whilst PPFD was almost three times higher in JIC chambers. Pots inside of chambers were rotated approximately once a week to minimise likely differences in microenvironments at different positions.

Treatments consisted of factorial combinations of different *Eps* alleles and different regimens of temperature. Temperature treatments of 24, 21, 15, 9 and 6°C were conducted at the UdL´s chambers and the temperatures regimens of 18 and 12 °C were tested at JIC´s chambers. All the temperatures were constant during day and night. Plants used in these experiments were two near isogenic lines (NILs) pairs segregating for *Eps* genes. Near isogenic lines were developed at John Innes Centre (Norwich. UK) from cross Spark × Rialto with *Eps* effect on chromosome 1DL. Each NIL pair was composed of a NIL carrying *Eps*-late allele and another carrying *Eps*-early allele.

In all the experiments, weeds, diseases and insects were controlled through spraying herbicides, fungicides and insecticides as recommended by their manufacturers. Furthermore, the experiments were irrigated when it was needed to avoid water stresses.

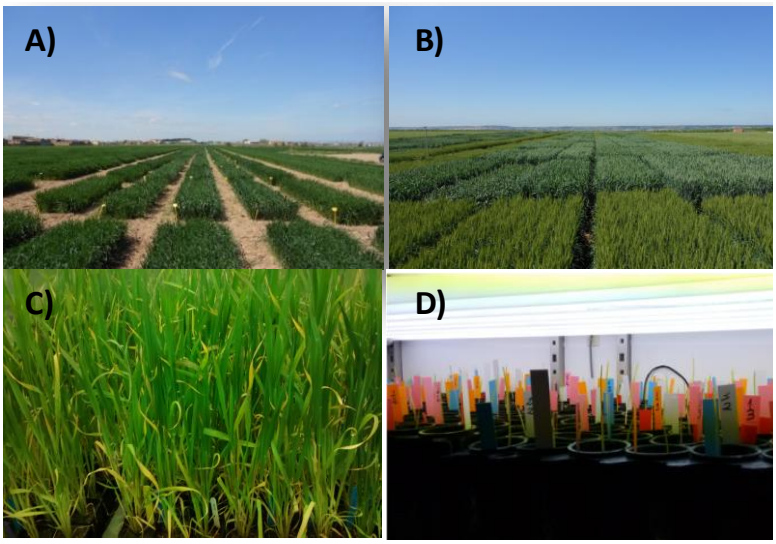

Fig. 2.1. Illustrations of some experiments carried out on this Thesis, Exp I (A), Exp V (B), Growth chambers of John Innes Centre (C) and Growth chambers of University of Lleida (UdL) (D).

2.2. Measurements and analysis

2.2.1. Developmental stages

Phenological stages were determined according to the decimal code developed by Zadoks *et al*. (1974): Onset of stem elongation (DC31) was established in plants with the first node detectable, flag leaf (DC39) was determined when flag leaf was fully unrolled with ligule just visible, heading (DC59) was measured in plants with the full spike outside the sheath of flag leaf in the main shoot and anthesis (DC65) was considered when 50% anthers of spike were matured. Timing of each phenological stage in each experimental unit was recorded when 50% plants of each plot reached the specific stage.

Duration of developmental phases was expressed in thermal time, considering the average temperature that was recorded by meteorological stations from the agro-meteorological network of Catalonia and assuming a base temperature of 0°C. In experiment VIII (the growth chamber experiment) the duration of developmental phases was analysed in calendar days in order to explore cardinal temperatures (base, optimum and maximum temperatures) through the ontogenic phases.

2.2.2. Final leaf number and dynamics of leaf appearance

At seedling emergence, three plants per experimental unit were selected and labelled in order

to monitor the number of leaf until flag leaf stage (DC39), stage in which the last leaf appears. Dynamics of leaf appearance were obtained by plotting the cumulative number of leaves appeared in the main shoot (Haun stage, Haun, 1973) against thermal time (Fig. 2.2). Data distribution provided a clear evidence of bilinear trends (early leaves appeared at a faster rate than late leaves), and therefore data were fitted through bilinear regression (Fig. 2.2). Phyllochron; i.e. thermal time elapsed between the appearance of two successive leaves, for the early and late appearing leaves was estimated as the reciprocal of the slopes of the reciprocal bi-linear regressions (thermal time *vs* emerged leaves) as doing so, instead of simply computing the reciprocal of the slope of the dynamics of leaf appearance, we obtained not only the values of phyllochron but also their standard errors and could then establish whether differences in this trait between isogenic lines were significant or not. The timing of the break in rate of leaf appearance was fitted to coincide with the emergence of the 7th leaf. This assumption is based on reports the change in phyllochron occurs when the Haun stage is between 6 and 8 (e.g. Jamieson *et al.*, 1995; Calderini *et al.*, 1996; Slafer and Rawson, 1997; González *et al.*, 2005) (Fig. 2).

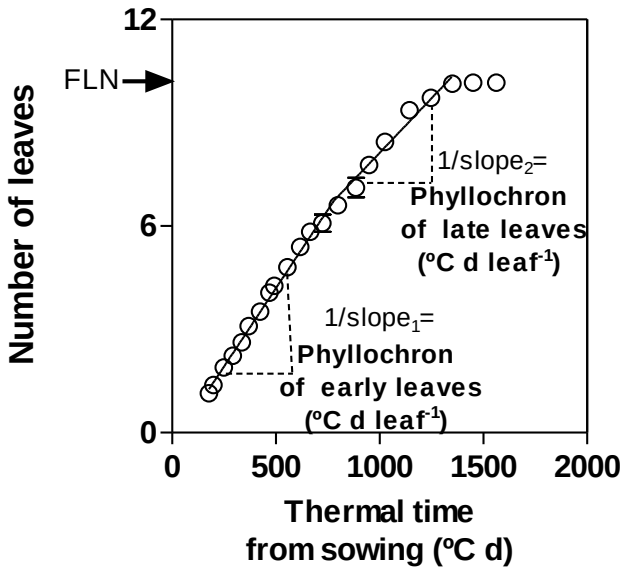

Fig. 2.2. Example of relationship between the number of appeared leaves in the main shoot and time after sowing fitted by bilinear regression. FLN: final leaf number.

2.2.3. Spikelet number and dynamics of leaf and spikelet initiation

One representative plant per experimental unit was randomly sampled and taken to the lab, where all these plants were dissected under a binocular microscope (Leica MZ 7.5, Leica

Microsystems, Heerbrugg, Switzerland) to (i) determine the developmental stage of apex according to Waddington *et al*. (1983) and (ii) count total number of leaves and spikelet primordia. With these records the dynamics of leaf and spikelet primordia initiation were analysed (and leaf and spikelet plastochrons -thermal time elapsed between the initiation of two successive primordia in the apex- were estimated as the reciprocal of the rate of primordia production). Dynamics of primordia initiation where obtained by plotting the number of primordia against thermal time. Data distribution had bilinear trends, being fitted by bilinear regression; the first phase in which initiation of leaf primordia are being generated was much slower than the second phase where spikelet primordia are being formed. Bilinear model was fitted with a fixed intercept (4 primordia, corresponding to the 4 leaves initiated during seed filling in the mother plant and therefore already developed in the embryo of the seeds sown). Time to floral initiation was calculated as time in which the total number of primordia of the apex exceeded the final leaf number emerged on the main shoot(González *et al.*, 2002) (Fig. 2.3).

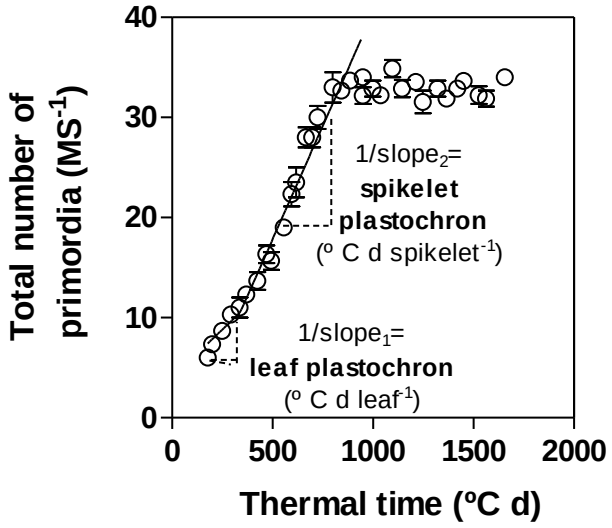

Fig. 2.3. Example of relationship between the total number of primordia and thermal time fitted by bilinear regression.

Double ridge and terminal spikelet stage was determined under binocular microscope in the dissection process when apex presented the value of 2.5 and 3.5 according to Waddington *et al*.(1983) (Fig. 2.4).

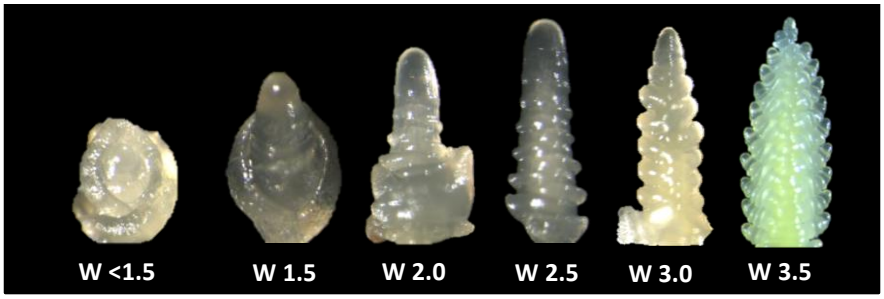

Fig. 2.4. Illustration of apex development from seedling emergence to terminal spikelet stage (W 3.5).

To analyze the coordination between leaf+spikelet primordia initiation and number of emerged leaf, the relationship between total number of primordia and number of emerged leaves on the main stem was explored. This relationship presented a bilinear trend and was fitted by a bilinear model with fixed intercept at 6 total of primordia, because in seedling emergence, the plants present two visible leaves and the embryo of the mature grain had already initiated four primordia, resulting in a total of 6 primordia. The number of spikelets of a particular line was counted in each dissection from terminal spikelet to anthesis for each experimental unit. The average of all these values determined the number of spikelet per spike for each genotype and environmental condition. Furthermore, at anthesis, four plants per each genotype and experimental unit was sampled and the final spikelet number was counted on main shoots and in all the tillers presented in these four plants.

2.2.4. Fertile tillers and dynamics of tillering

Dynamics of tillering (including tiller mortality) were analysed using a tetralineal model, as in Alzueta *et al.* (2012). This model was fitted by a curvilinear model because it yields parameters with both biological meaning and agronomic relevance. It has an intercept indicating the onset of tillering, a first upward slope representing the rate of tillering, the maximum number of tillers and the timing when this occurs, the onset of tiller mortality, the rate of tiller mortality and lastly the final number of living tillers, which will become the number of spikes (Fig. 2.5). To assess any possible effects on the coordination between tillering and leaf emergence, tiller dynamics was also analysed against the number of emerged leaves, determining the onset of tillering (in terms of number of leaves, or phyllochrons) and tiller appearance rate per emerged leaf.

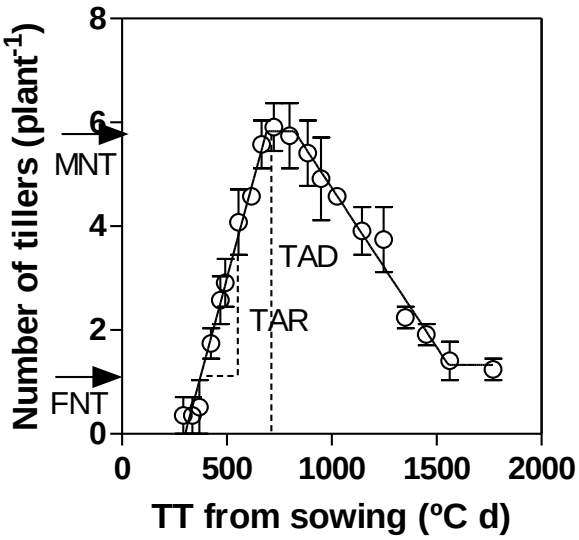

Fig. 2.5. Example relationship between the number of tillers and time after sowing fitted by tetralinear regression. TAR: tiller appearance rate, TAD: the duration of tillering, MNT: the maximum number of tillers reached at the end of tillering (as the average of the values determined from then to the onset of tiller mortaility), and FNT: the final number of tillers which are the number of fertile (spike-bearing) tillers.

2.2.5 Statistical analysis

All analyses were performed using statistical software JMP® Pro Version 12.0 (SAS Institute Inc. Cary, NC, USA) in order to determine the significance of differences between genotypes. Differences between genotypes were tested using ANOVA and a *post-hoc* analysis of LSMeans Contrast and of Student´s *t* test with significance at $P<0.05$. The model established for each experimental Chapter, considered the experimental design and the factors involved (as random or fixed factors).

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Chapter III

Duration of developmental phases, and dynamics of leaf appearance and tillering, as affected by source and doses of photoperiod insensitivity alleles in wheat under field conditions

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3. Chapter III: Duration of developmental phases, and dynamics of leaf appearance and tillering, as affected by source and doses of photoperiod insensitivity alleles in wheat under field conditions

3.1. Introduction

Variation on flowering time allows the adaptation of crops to particular environmental conditions. Genetic control of flowering time (anthesis) in wheat is mainly regulated by photoperiod, vernalisation and "earliness *per se*" genes (Slafer *et al*., 2015). Photoperiod response is determined by allelic variation at *Ppd-A1*, *Ppd-B1* and *Ppd-D1* genes (formerly *Ppd3*, *Ppd2* and *Ppd1*, respectively) located on the short arm of each homoeologous group 2 chromosome (Beales *et al*., 2007). Photoperiod insensitive wheats flower independently of day length, whilst sensitive wheats delay anthesis when grown under short days (Bentley *et al*., 2011). Photoperiod insensitive alleles are denoted by adding an *a* suffix, whilst photoperiod sensitive alleles carry a *b* suffix (McIntosh *et al*., 2003).

Alleles of *Ppd-1* are largely responsible for crop adaptation to mega-environments (Griffiths *et al*., 2009). In general, *Ppd-D1a* shows the strongest effect in reducing time to anthesis (e.g. Beales *et al*., 2007; Jones *et al*., 2017; Kiss *et al*., 2014; Snape *et al*., 2001; Yang *et al*., 2009; Worland, 1996). But the mode of action of the three *Ppd* genes are different. For instance, Tanio and Kato (2007) and Bentley *et al*. (2011) found that *Ppd-B1a* and *Ppd-A1a* also reduced time to anthesis, and Stelmakh (1998) reported that in their study *Ppd-A1a* showed stronger insensitivity than *Ppd-1* insensitive alleles of the B and D genome. As the ranking in strength of the particular *Ppd* genes is not universal, the source (donors) of the particular insensitivity alleles may play a significant role, beyond the particular genome in which it is introgressed. However, the effect of the source of alleles is largely undocumented. In general, the number of *Ppd-1a* alleles introgressed in the genome (doses) is accepted to be positively related with the strength of the reduction in time to anthesis (i.e. *Ppd* insensitivity alleles have additive effect). But this does not always happen: e.g. under constant short days, lines with two *Ppd* insensitivity mutations flowered earlier than lines with photoperiod insensitivity in only one genome, but not later than lines with insensitive alleles on all three genomes (Shaw *et al*., 2012). There are almost no studies comparing simultaneously lines with different doses of *Ppd-1a* alleles, and with different sources of some of the alleles which prevents concluding on the

relative importance of the *Ppd* alleles, the doses and the source of the alleles in accelerating development towards anthesis under natural photoperiods in field grown plots. Most of what we know on the effects of *Ppd* genes on adaptation relates to the effects on time from sowing or seedling emergence to anthesis, considering it as a single whole period. However, time to anthesis is composed of different developmental phases in which leaves, spikelets and florets, that will be later sources and sinks determining yield, are being formed (Slafer and Rawson, 1994). These are the vegetative (when leaf primordia are generated), the early reproductive (when spikelet primordia are formed) and the late reproductive phase (when firstly initiation and then degeneration of floret primordia take place). Evidence of genetic variation in duration of these phases as well as of environmental effects on them has been reported (Brown *et al*., 2013; García *et al*., 2011; Kirby *et al*., 1999; Sanna *et al*., 2014; Slafer and Rawson, 1994). Attempts have been made to identify QTLs related to the duration of specific sub-phases within the variation available in mapping populations (e.g. Borràs-Gelonch *et al*., 2012; Sanna *et al*., 2014). It has been far less documented to what degree *Ppd* alleles affect the duration of the phases composing time to anthesis (e.g. González *et al*., 2005b; Whitechurch and Slafer, 2002) and in these few cases the number of isogenic lines used was rather limited. In particular, the effect of different sources of specific *Ppd* alleles or dosage of *Ppd* genes on the duration of these sub-phases of time to anthesis are, to the best of our knowledge, non-existent. Recognising the effects of particular alleles on vegetative and reproductive phases is relevant not only academically but also potentially useful empirically. This is because there is a compelling range of evidences that whilst crop growth during the late reproductive phase is critical for yield, reducing growth during the vegetative and early reproductive phases does not affect it (e.g. Abbate *et al*., 1997; Demotes-Mainard and Jeuffroy, 2004; Ferrante *et al*., 2012; Fischer, 1985; Fischer, 2011; Fischer, 2016; González *et al*., 2005a; Savin and Slafer, 1991; Slafer, 2003). Therefore, it could be potentially useful for achieving genetic gains in yield to manipulate the durations of vegetative, early reproductive and late reproductive phases (Slafer *et al*., 2001; Miralles and Slafer, 2007), which could be facilitated if effects of particular alleles on specific phases composing time to anthesis were uncovered.

Photoperiod affects final leaf number (FLN) on main shoots of sensitive genotypes (e.g. Brooking *et al*., 1995) and in many cases most of the effect on time to heading or anthesis was explained by the effect on FLN (e.g. Brooking *et al*., 1995; Brooking and Jamieson, 2002; Slafer and Rawson, 1995a). This is in line with the pioneering work of Cooper

(1956) who found that photoperiod sensitivity in wheat started from the appearance of first leaf, therefore not possessing a juvenile phase (e.g. Hay and Kirby, 1991; Slafer and Rawson, 1995a). In some models FLN was used to predict time to anthesis, assuming that phyllochron (reciprocal of rate of leaf emergence or the thermal time interval between the emergence of two consecutive leaves) is constant (e.g. Cao and Moss, 1989). However, some studies demonstrated that phyllochron may vary in response to photoperiod particularly that of the later leaves (Slafer and Rawson, 1997). To what degree the effect of *Ppd* genes on time to anthesis might also be mediated through changes in phyllochron has been much less studied. The effects of *Ppd* genes on phyllochron may be relevant beyond its effects on time to anthesis. Tillering is critical for rapidly intercepting resources by the crop in early stages and determines the final spike number, which is a key component of yield. Tillering is controlled by both genetic and environmental factors (Assuero and Tognetti, 2010; Xie *et al*., 2016). Furthermore, tillering dynamics are related to the dynamics of leaf appearance (Kirby *et al*., 1985), but the degree of coordination between the appearance of tillers and leaves may be not constant (Alzueta *et al*., 2012). Therefore, it is possible that genes affecting phyllochron may also affect tillering dynamics. This likely effect of *Ppd* alleles has not been considered so far.

Therefore, the aim of this work was to quantify the impact of photoperiod insensitivity alleles and homoeoalleles, one of them from different donors, when introgressed in the A, B and/or D genomes of a photoperiod sensitive spring wheat variety on (i) the duration of vegetative and reproductive phases and (ii) the dynamics of appearance of leaves and tillers in field grown wheat near isogenic lines for *Ppd* genes.

3.2. Materials and methods

3.2.1. Experimental field conditions

Two field experiments were carried out during the 2012/13 and 2013/14 growing seasons nearby Bell-lloc d´Urgell (41.63°N, 0.78°E) in Catalonia, North-East Spain. In both experiments the soil was classified as a complex of *Calcisol petric* and *Calcisol haplic*, (FAO, 1990). Experiments were sown on 24 November 2012 and 12 November 2013 at a rate of 300 seeds m^{-2}. To minimise the experimental error, a week after emergence we labelled in each plot areas in which samples would have be taken later on in the season. In those areas we aimed to have 240 emerged seedlings per m^2, and when necessary we

thinned by hand these areas to have that density with very high uniformity. To double-check this we counted number of adult plants at anthesis (that were 238±5 and 247±4 plants per m^2 in the first and second growing season, respectively). Weeds, diseases and insects were controlled through spraying herbicides, fungicides and insecticides as recommended by their manufacturers.

The mean daily global radiation (MJ m^{-2}), maximum and minimum temperature (°C) and accumulated precipitation (mm) were recorded daily by meteorological stations from the agro-meteorological network of Catalonia. Meteorological stations were located close to the experimental fields. Averaging across the growing season, temperature was *c*. 1ºC warmer in the second than in first growing season (which tended to be cooler than the average of the six previous years; Fig. 3.1). This difference in temperature between seasons was more marked in April and May (months in which the crop was reaching anthesis stage in all lines) than in earlier parts of the growing season. These particular months were clearly warmer (*c*. 3ºC) in the second than in the first growing season (Fig. 3.1). Although both growing seasons had a higher accumulated precipitation than the average of the six previous years (264.3 and 303.0 mm for first and second growing season, respectively compared with the average of six previous years 246.5 mm), in order to avoid water stress in critical stages of development, rainfall was supplemented with irrigation. In the first growing season, that had a wetter spring, only one irrigation was given on 16 April 2013. In the second, drier, growing season the field was irrigated both on 26 March and 3 May 2014. Each irrigation consisted of 80 l m^{-2}.

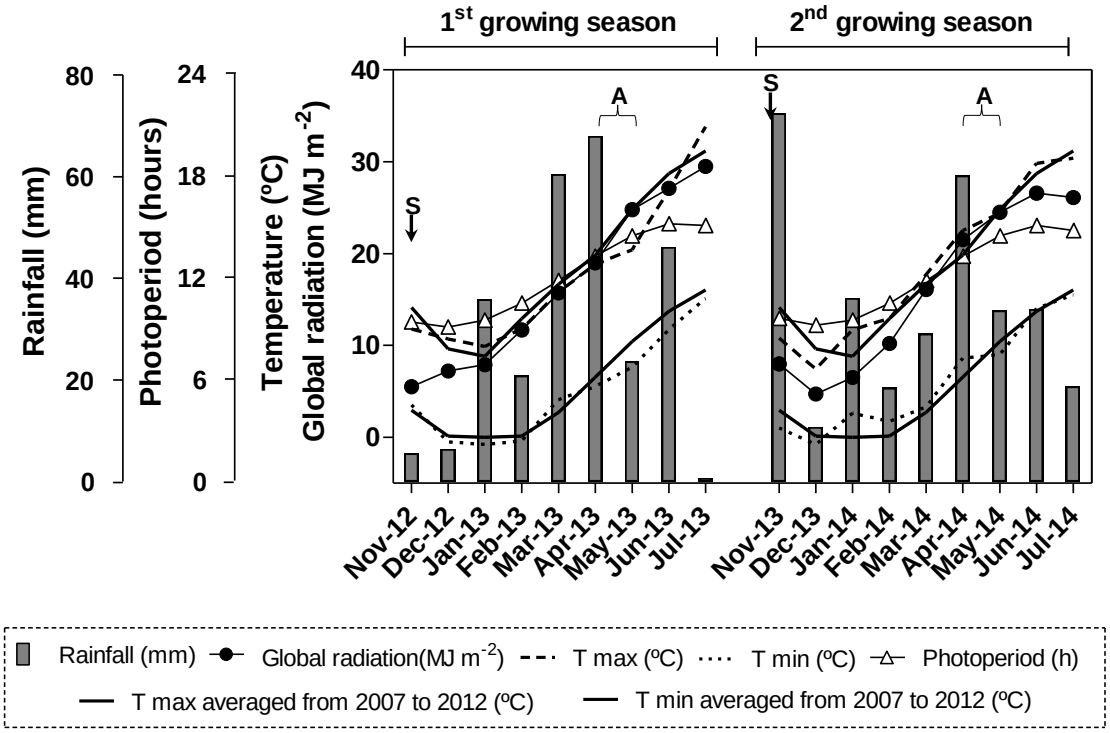

Fig. 3.1. Accumulated rainfall, maximum and minimum temperature, photoperiod and global incident radiation for monthly periods during the two growing seasons (2012/13, left and 2013/14, right). The arrow with the "S" indicates sowing date and the key with the "A" shows the range of dates in which anthesis was reached.

3.2.2. Treatments and design

Treatments consisted of 12 near isogenic lines (NILs; i.e. lines differing in genes of interest but with the same genetic backgrounds) carrying *Ppd-1a* alleles of hexaploid wheat developed at the John Innes Centre (Norwich, UK), according to Shaw *et al*. (2013). The 13 experimental genotypes were the "wild type" Paragon (a wheat cultivar with a strong sensitivity to photoperiod but insensitive to vernalisation) and 12 NILs in which *Ppd* alleles were introgressed on chromosome 2 of the A, B and/or D genomes of the wild type (Table 3.1).

A sample of the *Ppd-1* homoeoallic was represented by these alleles (i) *Ppd-A1a* from "GS-100" (carrying a 1027 bp promoter deletion) (Wilhelm *et al*., 2009); (ii) *Ppd-B1a* from either "Chinese Spring" (characterised by one truncated copy and three intact copies in tandem in a 185 kb region), "Sonora 64" (that has three intact copies in tandem), or from "Recital 64" (that presents two intact copies in tandem) (Beales *et al*., 2007); and (iii) *Ppd-D1a* from "Sonora 64" (that has a 2089 bp promoter deletion) (Beales *et al*., 2007).

Table 3.1. Near isogenic lines (NILs) with introgressed *Ppd* alleles (indicating the source of insensitive alleles introgressed into the wild type) and the wild type Paragon used in this study. The dose (number of photoperiod insensitivity alleles) introgressed in the wild type, indicating the *Ppd* alleles in each chromosome, and code to name the individual lines throughout this paper are shown (to facilitate the visual impact insensitive alleles are shown in bold type).

Genotypes (Lines; source of "*a*" alleles)	Dose of insensitive alleles	*Ppd* alleles	Code[1]
Paragon (wild type)	0	*Ppd-A1b*, *Ppd-B1b* and *Ppd-D1b*	A_P+B_P+D_P
GS-100 2A	1	***Ppd-A1a***, *Ppd-B1b* and *Ppd-D1b*	$\mathbf{A_{GS}}$+B_P+D_P
Chinese Spring 2B		*Ppd-A1b*, ***Ppd-B1a*** and *Ppd-D1b*	A_P+$\mathbf{B_{CS}}$+D_P
Sonora 64 2B		*Ppd-A1b*, ***Ppd-B1a*** and *Ppd-D1b*	A_P+$\mathbf{B_S}$+D_P
Recital 2B		*Ppd-A1b*, ***Ppd-B1a*** and *Ppd-D1b*	A_P+$\mathbf{B_R}$+D_P
Sonora 64 2D		*Ppd-A1b*, *Ppd-B1b* and ***Ppd-D1a***	A_P+B_P+$\mathbf{D_S}$
GS-100 2A+CS 2B	2	***Ppd-A1a***, ***Ppd-B1a*** and *Ppd-D1b*	$\mathbf{A_{GS}}$+$\mathbf{B_{CS}}$+D_P
GS-100 2A+ Sonora 64 2B		***Ppd-A1a***, ***Ppd-B1a*** and *Ppd-D1b*	$\mathbf{A_{GS}}$+$\mathbf{B_S}$+D_P
GS-100 2A+ Sonora 64 2D		***Ppd-A1a***, *Ppd-B1b* and ***Ppd-D1a***	$\mathbf{A_{GS}}$+B_P+ $\mathbf{D_S}$
Chinese Spring 2B+ Sonora 64 2D		*Ppd-A1b*, ***Ppd-B1a*** and ***Ppd-D1a***	A_P+$\mathbf{B_{CS}}$+$\mathbf{D_S}$
Sonora 64 2B +Sonora 64 2D		*Ppd-A1b*, ***Ppd-B1a*** and ***Ppd-D1a***	A_P+$\mathbf{B_S}$+ $\mathbf{D_S}$
GS-100 2A+ Chinese Spring 2B+Sonora 64 2D	3	***Ppd-A1a***, ***Ppd-B1a*** and ***Ppd-D1a***	$\mathbf{A_{GS}}$+$\mathbf{B_{CS}}$+$\mathbf{D_S}$
GS-100 2A+Sonora 64 2B+Sonora 64 2D		***Ppd-A1a***, ***Ppd-B1a*** and ***Ppd-D1a***	$\mathbf{A_{GS}}$+$\mathbf{B_S}$+$\mathbf{D_S}$

[1]this code denotes the genomes with sensitivity- (in plain text) or insensitivity-alleles (in bold type), indicating the source of such alleles (subscript; P=Paragon, GS=GS 100, CS=Chinese Spring, S=Sonora 64). In the case of the sensitive alleles the source was always the wild type Paragon.

All in all, there were five NILs with only one insensitive allele introgressed, another five NILs with two insensitive alleles and two NILs with the three insensitive alleles, and for some of the cases alternative insensitivity alleles on the B genome from different source cultivars (Table 3.1). For the purpose of simplicity we refer to particular lines using a code considering whether the allele was sensitive or insensitive in each genome (plain font or bold font when designating the genome, respectively) and the source of the allele as a subscript (Table 3.1).

Treatments were arranged in a complete randomised design with different number of replications (ranging from one to five replications, depending on availability of seeds) in 2012/13 and in a randomised complete block design with three replications in 2013/14 Experimental plots measured 1.2 x 4 m. Row spacing was 0.2 m.

3.2.3. Measurements and analyses

Phenological stages of seedling emergence (stage DC10), flag leaf emergence (stage DC39) and anthesis (stage DC65) were determined according the Decimal Code developed by Zadoks *et al*. (1974). Two other phenological stages were critical for this study: the timing of floral initiation and of terminal spikelet. To determine these stages, plants sampled at random from each plot were frequently dissected, and the accumulated number of primordia was recorded. The timing of floral initiation measured in thermal time was determined *a posteriori* as the time when the number of primordia initiated in the apex exceeded the final number of leaves emerged on the main shoots (see below). Timing of terminal spikelet was determined as in Waddington *et al*. (1983). With these data the duration of the whole cycle to anthesis as well as that of its component phases (vegetative, until floral initiation; early reproductive, from then to terminal spikelet; and late reproductive, from terminal spikelet to anthesis) were computed in thermal time (thermal time was calculated as mean air temperature and with base temperature of 0°C, Kirby *et al*., 1985) for each plot.

At seedling emergence, three plants per plot were selected to represent the particular treatment, minimising the noise from changes in the competitive conditions within and among plots, which could be relevant for tillering dynamics. These plants have to be (i) at the expected plant density in its surrounding area, (ii) uniformly distributed, and (iii) emerged when 50% of emergence of the plot occurred. For that purpose we selected within a week after emergence these three plants in that condition within each plot and labelled them individually. From then on, we monitored leaf and tiller appearance. From seedling

emergence to anthesis, the number of leaves appeared on the main shoot (Haun, 1973) and the number of shoots were recorded once or twice weekly, depending on temperature on these plants. After the appearance of 3-4 leaves, the last expanded leaf in that main shoot was marked to avoid miscounting the accumulated number of leaves due to senescence of the oldest or not recognising the main shoot after the onset of tillering. Throughout the cycle, from seedling emergence to anthesis there were 21 determinations of leaf and tiller number in each of the three plants measured. Means were calculated for each sampling date and plot. No distinction was made between tiller categories, only the total number of tillers. With these values the dynamics of leaf appearance and tillering were analysed for each line.

Dynamics of leaf appearance were obtained by plotting the cumulative number of leaves appeared in the main shoot (Haun, 1973) against thermal time. Although the coefficient of determination for the linear relationship between leaf number and time was statistically significant in all cases, data distribution provided clear evidence of bilinear trends (early leaves appeared at a faster rate than late leaves), and therefore data were fitted using bilinear regression. Phyllochron for the early and late appearing leaves was estimated as the reciprocal of the slopes of the reciprocal bi-linear regressions (thermal time *vs* emerged leaves). So, instead of simply computing the reciprocal of the slope of the dynamics of leaf appearance, we obtained the values of phyllochron together with standard errors which we could then use to establish whether differences in this trait between lines were significant or not. The timing of the break in rate of leaf appearance was fitted to coincide with the emergence of the 7^{th} leaf. This assumption is based on reports the change in phyllochron occurs when the Haun stage is between 6 and 8 (e.g. Calderini *et al*., 1996; González *et al*., 2005b; Jamieson *et al*., 1995; Slafer and Rawson, 1997); and assuming that this threshold is applicable across a very wide range of conditions (Miralles *et al*., 2001). Thus, the phyllochron of early leaves refers to the pace of leaf appearance corresponding to the first 7 leaves, whilst phyllochron of late leaves refers to that of leaves appearing from then to the flag leaf.

Dynamics of tillering (including tillering and tiller mortality) were analysed using a tetralineal model, as in Alzueta *et al*. (2012). It has an intercept indicating the onset of tillering, a first upward slope representing the rate of tillering, the maximum number of tillers and the time when this occurs, the onset of tiller mortality, the rate of tiller mortality and lastly the final number of living tillers, which will become the number of spikes. To assess any possible effects on the coordination between tillering and leaf emergence, tiller dynamics was also

analysed against the number of emerged leaves, determining the onset of tillering (in terms of number of leaves, or phyllochrons, at the onset of tillering) and tiller appearance rate per emerged leaf.

A mix model was fitted for all the analysed traits using statistical software JMP® Pro Version 12.0 (SAS Institute Inc. Cary, NC, USA). Growing seasons, genotypes, and their interaction were considered fixed, whereas blocks nested within growing seasons were identified as random. Differences between *Ppd-1* alleles were tested using analysis of LSMeans Contrast and of Student´s *t* test with significance at $P<0.05$.

3.3. Results

There were large environmental (growing seasons) and genotypic differences in most variables measured, indicating the major effect of *Ppd-1a* alleles had on developmental patterns of wheat. These genotypic effects were not restricted to those in total time to anthesis and final leaf number, which are well known, but also highly significant for each of the component phases of time to anthesis (Table 3.2). The genotype by growing season (GxGS) interaction was also statistically significant in most of the traits analysed; but its magnitude was always smaller than that of the genotypes (Table 3.2). Considering the two most integrative traits analysed (time to anthesis and final leaf number), the magnitude of the mean squares corresponding to the genotypic effects was between 10 and 25-fold larger than those of the GxGS interaction (Table 3.2). Although the magnitude of the difference was not that large, the mean squares of the genotypic effects were also consistently greater than those of the GxGS interaction for the duration of the three component phases determining the time to anthesis (Table 3.2).

Table 3.2. Mean squares of the effects of the growing season, the genotypes and their interaction on the main traits analysed: (i) thermal time from sowing to anthesis, (ii) duration of its three component phases (vegetative, early reproductive and late reproductive), and (iii) final leaf number.

Source of variation	df	Sowing to anthesis (ºC d)	Vegetative (ºC d)	Early reproductive (ºC d)	Late reproductive (ºC d)	Final leaf number (leaves)
Growing season	1	137,910***	9,679**	201,192***	95 ns	7.02***
Genotypes	12	39,811***	4,093***	11,861***	5,611***	1.25***
GxGS	12	1,517**	3,088***	5,252***	3,230**	0.13 ns

Asterisks indicate the significance level of the F-ratio (**P<0.05, **P<0.01, ***P<0.001*, and ns-non significant).

Therefore, even though the GxGS interaction was statistically significant in many cases, the interaction was not a crossover one: i.e. it was quantitative (the magnitude of the difference between genotypes with different *Ppd-1a* alleles was different between the two growing seasons) but not qualitative (the direction of the effect was the same in both seasons). This can be clearly illustrated with time to anthesis. The cycle was consistently longer in the second growing season but within growing seasons the genotypic effects were rather consistent, and the main reason for the significant GxGS interaction was that in the first season there was not a significant difference between NILs with 2 or 3 *Ppd-1a* alleles, whilst in the second growing season the difference was significant (Supplementary Fig. FS3.1).

Consequently, in spite of the fact that the GxGS interaction was statistically significant for most traits the effects of the doses, genomes and sources of *Ppd-1a* alleles can be reliably considered averaged across the two growing seasons.

3.3.1. Duration of time to anthesis

Genotypes carrying insensitive alleles took significantly less time to reach anthesis than wild type-Paragon (Fig. 3.2, left panels). In general, the larger the dose of insensitivity alleles the stronger the effect on reducing total time to anthesis (Fig. 3.2, left panels), although the acceleration of developmental rates produced by introgressing *Ppd-1a* alleles was less than proportional with the increase in doses: introgressing 1 allele reduced time to anthesis by *c.* 190ºC d whilst each extra doses advanced anthesis by *c.* 60ºC d more (Fig. 3.2, left panels).

The strength in reducing time to anthesis of *Ppd-1a* alleles in each particular genome exhibited a clear trend for the insensitivity in the A and D genome to be stronger than that in the B genome (strength of the alleles was *Ppd-D1a*≥*Ppd-A1a*>*Ppd-B1a*, where ≥ indicates that the effect was stronger arithmetically but the difference was not statistically significant at *P<0.05* (Fig. 3.2, top middle panels). When comparing the cases in which two doses were introgressed, the stronger effect of insensitivity from the A and D genomes, compared with that in B was confirmed: insensitivity from A and D homoeoalles together was stronger than that conferred by combinations of insensitivity homoeoallele carried on the A and B genomes or the B and D genomes (Fig. 3.2, bottom middle panels). When comparing allelic effects we

fully acknowledge the possibility that alleles linked at the introgressed locus and random background segments maintained in spite of five rounds of backcrossing (~3% donor background expected) might contribute to variation in the observed traits. Although this could be reduced by further backcrossing and the identification of close recombinants it is a question always likely remain using traditional genetic approaches. For future work we propose the use of gene editing technologies to recreate these alleles with no linkage or background effects (Doudna and Charpentier, 2014; Kumar and Jain, 2014; Ran, 2014).

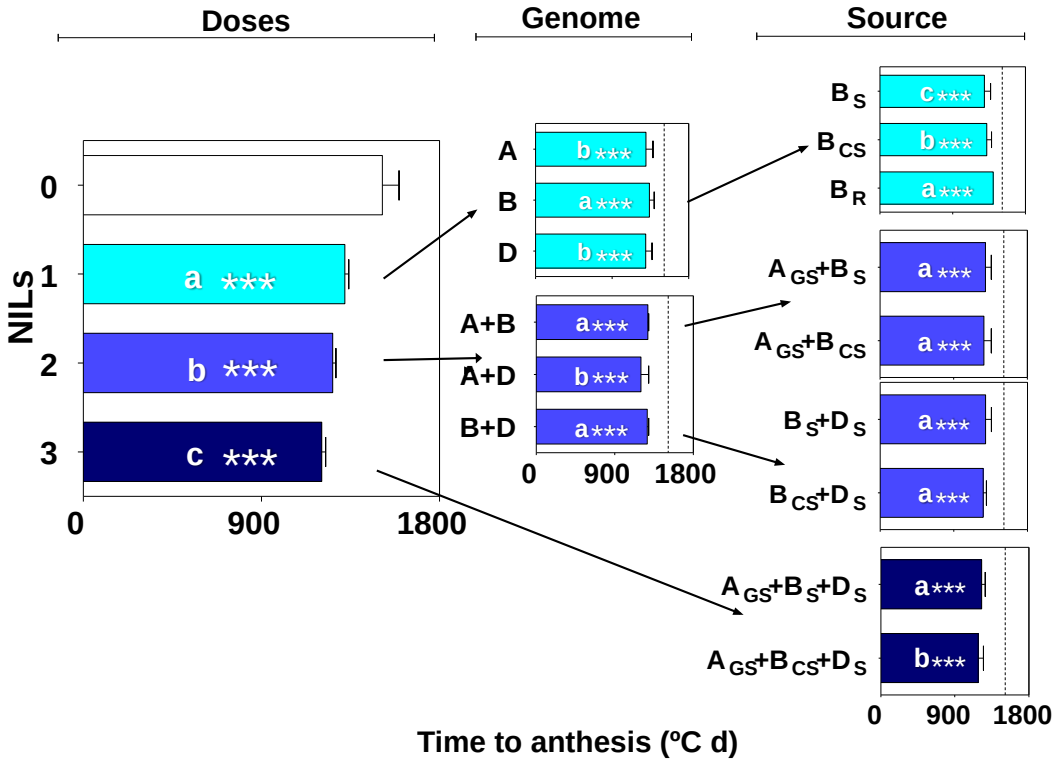

Fig. 3.2. Duration of the period from sowing to anthesis of the different *Ppd-1a* NILs and the wild type-Paragon (open bar on the left panels, dotted lines in all other panels). On the left the durations are averaged across lines with the same doses of insensitive *Ppd* homoeoalleles including the wild type Paragon with all three alleles sensitive to photoperiod. In the middle, the durations are identified for the genomes in which the insensitivity was introgressed, in the case of the B genome averaged across the different sources of the allele. On the right the durations are shown for each of the different sources used as donors of *Ppd-B1a* alleles. Asterisks indicate the statistical significance of the differences between lines with insensitivity alleles and Paragon from the LSmeans contrast (**P<0.05*, ***P<0.01*, ****P<0.001* and ns-non significant). Different letters within bars indicate that the differences between lines with different doses of *Ppd-1a* alleles (left), or between lines with different genomes with insensitivity, within doses (middle), or between lines with different sources of *Ppd-B1a* alleles, within doses and genomes (right) are statistically significant (*P<0.05*). Segments in each bar stand for the standard error of the means used to calculate the time to anthesis in each case.

To take into account the effect of the source of the *Ppd-1a* alleles within the same genome, we

explored three different sources for the *Ppd-B1a* alleles. *Ppd-B1a* from Chinese Spring was stronger than that from Recital but weaker than that from Sonora 64 (Fig. 3.2, top right panels). Thus the actual ranking of strength of *Ppd-A1a*, *Ppd-D1a*, and *Ppd-B1a* may well depend more on the source of the specific *Ppd-1a* allele, including flanking genes, than on the genome. Ranking the strength of the studied *Ppd-1a* alleles for the NILs with only one dose was *Ppd-B1a*$_S$≥*Ppd-D1a*≥*Ppd-A1a*≥*Ppd-B1a*$_{CS}$>*Ppd-B1a*$_R$, indicating that *Ppd-B1a* could be the strongest or the weakest of all the *Ppd-1a* analysed in the present study, depending on the source of the allele. For all of the B genome alleles insensitivity is the result of increased copy number. The earliness of *Ppd-B1a*$_S$ compared to *Ppd-B1a*$_R$ might simply be due to three intact copies versus two. However, *Ppd-B1a*$_{CS}$ carries three intact copies and one truncated. Diaz *et al*., 2012 found a similar ranking for the *Ppd-B1a* alleles in the same lines grown under short days. Considering the double and triple insensitive lines, we used two different sources, and in the case of the lines with two *Ppd-1a* alleles introgressed there was no difference depending on whether the *Ppd-B1a* was from Chinese Spring or Sonora 64 (Fig. 3.2, middle right panels). And, when considering the NILs with *Ppd-1a* alleles in all three genomes, the strength reverted to what was found in the NILs with a single insensitivity allele: *Ppd-B1a* from Chinese Spring was stronger than that from Sonora 64 (Fig. 3.2, top bottom right panels), revealing that *Ppd-1a* homoeoalleles might display epistatic interaction effects.

3.3.2. Duration of vegetative and reproductive phases

The introgression of *Ppd-1a* alleles reduced significantly the duration of the vegetative phase respect to that of Paragon, but there was no clear effect of the doses: the reduction was similar with 1, 2 or 3 *Ppd-1a* alleles, *c*. 70ºC d (Fig. 3.3, top row left panel). The particular genome in which the *Ppd-1a* alleles were introgressed did not affect consistently the magnitude of the effect on the duration of the vegetative phase in the NILs with a single dose, whilst in the NILs with two alleles introgressed it seemed that the joint action of the alleles in genomes A and D were stronger than when the genome B was involved (Fig. 3.3, top row middle panels). Similarly, in the case of the total time to anthesis, the source of the allele seemed to have been paramount. The duration of the vegetative phase was significantly reduced when the *Ppd-B1a* alleles were obtained from Sonora 64 or Chinese Spring acting as donors, with no significant differences in strength between them, but it was not significantly affected when the *Ppd-B1a* allele came from Recital (Fig. 3.3, top row right panels). Therefore, a ranking of strength of

Ppd-1a alleles to reduce the duration of the vegetative phase, considering the NILs with a single dose, was *Ppd-B1a*$_S$≥*Ppd-B1a*$_{CS}$≥*Ppd-D1a*≥*Ppd-A1a*>*Ppd-B1a*$_R$.

On the other hand, when *Ppd-1a* alleles were introgressed in only one genome they did not affect significantly (*P=0.15*) the duration of the early reproductive phase (Fig. 3.3, middle row left panel). When the photoperiod insensitivity alleles were introgressed in two or three genomes simultaneously they reduced the length of this phase in 132°C d with respect to the wild type (with no differences between lines with two or three photoperiod insensitivity mutations introgressed). Analysing *Ppd-1a* alleles in each genome separately, it may be suggested that the overall effect of *Ppd-1a* alleles in one genome did not affect the early reproductive phase due to the fact that lines with *Ppd-B1a* or *Ppd-D1a* were similar to the wild type-Paragon, although *Ppd-A1a* shortened this phase (*P=0.03*)(Fig. 3.3, middle row middle panels). The lack of significant effects of a single dose of *Ppd-B1a* reflected a lack of significant effect of all three sources considered for this allele (Fig. 3.3, middle row right panels). Although *Ppd-B1a*$_S$ tended to have a stronger effect than A and D genome homoeoalleles when considering a single insensitivity allele, the analysis of the double and triple insensitivities did not confirm that trend (Fig. 3.3, middle row right panels). Ranking *Ppd-1a* alleles for their strength to reduce the duration of the early reproductive phase was *Ppd-A1a*>*Ppd-B1a*$_S$≥*Ppd-D1a*≥ *Ppd-B1a*$_{CS}$>*Ppd-B1a*$_R$, when considering the NILs with only one *Ppd-1a* allele introgressed.

When considering the late reproductive phase, again the introgression of *Ppd-1a* alleles reduced significantly the duration of the phase and the effect was similar with 1 or 2 *Ppd-1a* alleles introgressed (by *c*. 60ºC d), but when these alleles were in all the genomes the reduction (*c*. 90ºC d) was significantly stronger than when there were 1 or 2 doses (Fig. 3.3, bottom row left panel). The introgressed insensitive homoeoallele affected: the late reproductive phase, which was slightly reduced by *Ppd-A1a* (*P<0.10*), while it was more clearly reduced by *Ppd-B1a* and particularly *Ppd-D1a* alleles (Fig. 3.3, bottom row middle panels). The fact that lines carrying *Ppd-D1a* showed the strongest expression of this trait was consistent with results of the NILs with two doses, in which the strongest effect was observed when *Ppd-D1a* acted together with *Ppd-A1a* (followed by *Ppd-A1a + Ppd-D1a* and then *Ppd-A1a + Ppd-B1a*). When considering the effect of the source of the *Ppd-B1a* allele, when the donor was Recital this phase was not shortened. Reduction was most apparent when the insensitivity came from Chinese Spring and intermediate when the donor was Sonora 64, and

this trend was confirmed in most (though not all) the other NILs with 2 and 3 insensitivity alleles (Fig. 3.3, bottom row right panels). Therefore, a ranking of strength of *Ppd-1a* alleles to reduce the late reproductive phase, considering the NILs with a single dose, was *Ppd-D1a*≥*Ppd-B1a*$_{CS}$ ≥*Ppd-B1a*$_{S}$> *Ppd-A1a* >*Ppd-B1a*$_{R}$.

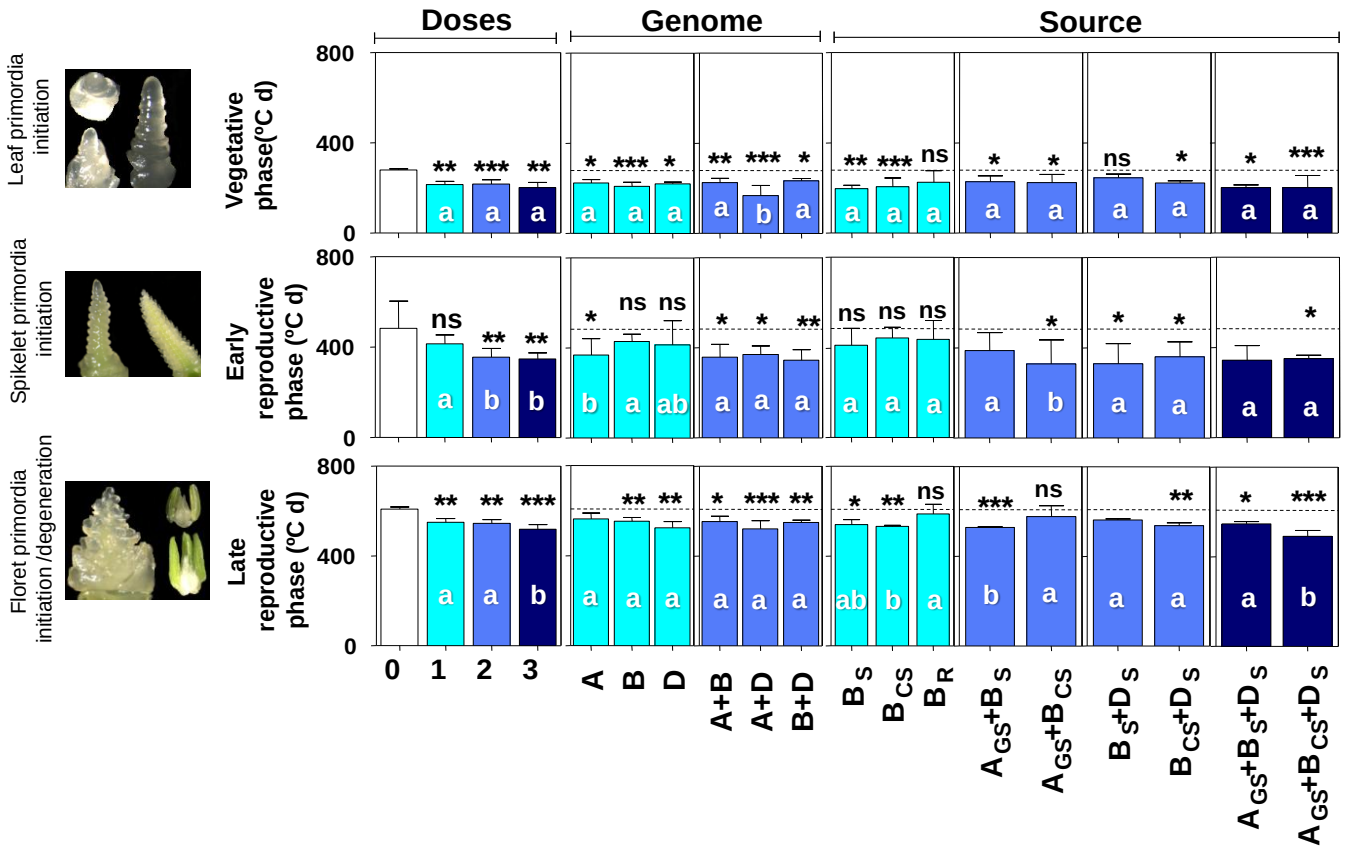

Fig. 3.3. Duration (in thermal time) of the three component phases of time to anthesis: the vegetative (from seedling emergence to floral initiation; top row of panels), the early reproductive (from floral initiation to terminal spikelet; middle row of panels) and the late reproductive (from terminal spikelet to anthesis; bottom row of panels) of different NILs and wild type- Paragon (open bars on left panels, dotted lines in other panels) averaged across growing seasons. Left panels represent the durations averaged across lines with the same doses of insensitive *Ppd* alleles including the wild type Paragon. In the intermediate panels, the durations are identified for the genomes in which the insensitivity was introgressed, in the case the B genome averaged across the different sources of the allele. In the right panels the durations are shown for each of the different sources used as donors of the *Ppd-B1a* allele. Asterisks indicate the statistical significance of the differences between lines with insensitivity alleles and Paragon from the LSmeans contrast (*$P<0.05$, **$P<0.01$, ***$P<0.001$ and ns-non significant, if nothing is noted on top of a bar it implies that $P<0.10>0.05$). Different letters within bars indicate that the differences between lines within panels are statistically significant ($P<0.05$). Segments in each bar stand for the standard error of the means used to calculate the durations in each case.

As when considering the whole set of NILs the insensitivity alleles reduced all the phases considered, the relationship between the total time to anthesis and the duration of each phase was in all cases positive and significant (Fig. 3.4, left panel). However, it seemed that overall combinations of doses, genomes and sources of *Ppd-1a* alleles the insensitivity to photoperiod accelerated development towards anthesis more through accelerating the early reproductive phase than the other two phases considered. This is not based on the marginally larger coefficient of correlation but on the clearly smaller slope, which implies that the absolute variation in duration of this phase was larger than that of the vegetative and late reproductive phase (Fig. 3.4, left panel). If the regressions are analysed reducing the variation from specific *Ppd-1a* alleles, averaging the variables across doses of these alleles introgressed, the proportion of the variation in time to anthesis explained by each of the component phases increased noticeably (Supplementary Fig. FS3.2), reinforcing the perception that all three component phases are similarly responsible for the response of time to anthesis to the introgression of *Ppd-1a* alleles.

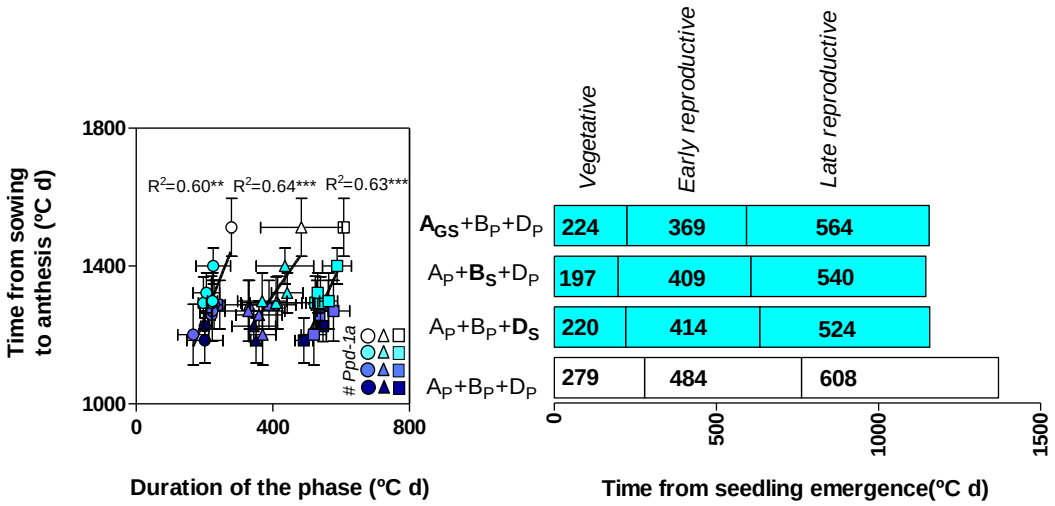

Fig. 3.4. Left panel: relationship between time from sowing to anthesis and the duration of the three component phases (vegetative, circles; early reproductive, tringles; and late reproductive, squares) for Paragon (open symbols) and the NILs with 1, 2 or 3 doses of *Ppd-1a* alleles (pale, intermediate and dark symbols). The coefficient of determination (R^2) and the level of significance (***P<0.01*, ****P<0.001*) were included. Right panel: duration of the three phases (vegetative, early reproductive and late reproductive, from left to right) in Paragon (open bars) and in three of the NILs with a single introgression of *Ppd-1a* alleles (pale bars). Figures have inside each phase stand for its duration in thermal time.

However, this observation only holds true when considering the effects of overall combinations of doses, genomes and sources of *Ppd-1a* alleles analysed in Fig. 3.4 (left

panel). More than 35% of variation in time to anthesis was associated with differences in durations of the phases produced by particular *Ppd-1a* alleles (Fig. 3.4, right panel). To illustrate this issue with a simple comparison we selected three cases with a single *Ppd-1a* allele introgressed in each of the three genomes in which time from seedling emergence to anthesis was similarly reduced (from *c*. 1370 in Paragon to *c*. 1150ºC d in each of these three NILs) but the partitioning of the time to anthesis into different component phases was quite different (Fig. 3.4, right panel). When the single dose of *Ppd-1a* introgressed was that from the genome A of GS-100 the reduction in time to anthesis was strongly linked to that of the vegetative (*c*. 20%) and early reproductive phases (*c*. 25%) while the late reproductive phase was only marginally reduced (<10% and only statistically significant at $P<0.10$; see Fig. 3.3). In contrast, when the allele was donated from the D genome of Sonora 64 the duration of the early reproductive phase was less reduced (*c*. 15% and not statistically significant; see Fig. 3.3) with the partitioning of accelerated development shifted to the late reproductive phase (*c*. 15% and highly significant; see Fig. 3.3). The effect of *Ppd-B1a* from Sonora 64 had intermediate effects on both reproductive phases and the strongest reduction in the duration of the vegetative phase (Fig. 3.4, right panel).

3.3.3 Final leaf number, phyllochrons and its coordination with anthesis

Photoperiod insensitivity alleles caused a decrease in final leaf number (FLN) that was significant in all NILs except one (Table 3.3). As doses of *Ppd-1a* alleles increased, the magnitude of the reduction in FLN increased as well (Table 3.3). However, there was not a consistent trend for the strength of *Ppd-1a* of the different genomes for reducing FLN. Analysing the cases with a single dose of these alleles, *Ppd-A1a* and *Ppd-D1a* exhibited the same FLN (*c*. 1 less leaf than Paragon) and *Ppd-B1a* showed more or less effect depending on the specific allele but taking these alleles together it can be said that *Ppd-B1a* had similar strength to that of the other two genomes (Table 3.3). Regarding the effect of the source of *Ppd-B1a* alleles a clear difference was seen in the single introgression NILs. *Ppd-B1a*$_R$ did not significantly reduce FLN, the reduction was significant at $P<0.05$ for *Ppd-B1a*$_{CS}$ and at $P<0.01$ for *Ppd-B1a*$_S$ (Table 3.3). However, when combined with *Ppd-A1a* and/or *Ppd-D1a* alleles in NILs with 2 or 3 doses of insensitivity the effect of the source of *Ppd-B1a* (Sonora 64 *vs* Chinese Spring) disappeared (Table 3.3). Analysing the photoperiod insensitivity alleles introgressed in

a single genome, the same ranking of strength was observed as that seen for time to anthesis: *Ppd-B1a*$_S$≥*Ppd-D1a*≥*Ppd-A1a*>*Ppd-B1a*$_{CS}$>*Ppd-B1a*$_R$. Consequently, most (>85%) of the effects of these alleles on time to anthesis were due to their effects on FLN (Fig. 3.5, left panel).

In order to explore whether the *Ppd-1a* alleles affected the dynamics of leaf appearance, in addition to their effects in the number of leaves that appeared before heading and anthesis, we plotted for each case the Haun stage against time, as illustrated for the insensitive wild type and for the NILs with extreme effects on FLN (Supplementary Fig. FS3.3): A_P+**B_R**+D_P, which reduced FLN marginally and not significantly and **A_{GS}+B_{CS}+D_S**, which produced the strongest reduction in FLN (Table 3.3). As can be seen in the extreme cases chosen for illustrating the dynamics of leaf appearance, although the coefficient of determination of a linear regression between leaf number and time would have been rather high (ranging from 0.976 to 0.995), using such regression would have not left the residuals distributed at random: in all cases a significant quadratic pattern between residuals and time were evident (Fig. FS3.3, middle panels). This quadratic component of the regression between residuals of Haun stage revealed clearly that the dynamics of leaf appearance are better represented using a bi-linear model, which does produce a random distribution of residuals (Fig. FS3.3, right panels). The use of bi-linear regressions increased the coefficient of determination of the relationships between leaf number and time, ranging from 0.978 to 0.998. The bi-linear regression used gave two slopes: quantifying the rate of leaf appearance of the early (first slope) and late leaves (second slope); and the reciprocal of these rates was the phyllochron for early and late leaves. The introgression of *Ppd-1a* alleles did not affect phyllochron of the early leaves (Table 3.3). The phyllochron of the late leaves was reduced by insensitivity to photoperiod, particularly with 2 and 3 doses of *Ppd-1a* alleles , whilst the effect of a single dose was smaller and only in few cases significant (although the average of all NILs with a single dose showed a phyllochron significantly shorter than Paragon; Table 3.3). Thus, in general, the higher the dose of insensitivity alleles the stronger the reduction in phyllochron of late leaves up to two doses, there was no significant difference between lines with double or triple insensitivity (Table 3.3). There was not a clear and consistent effect of any of the particular genomes in which the *Ppd-1a* alleles were introgressed or of the source from where the alleles were introgressed (Table 3.3).

Table 3.3. Phyllochron for early (Phyll I) and late leaves (Phyll II) and final leaf number (FLN) of the wild type, Paragon (A_P+B_P+D_P) and all the different *Ppd-1a* NILs, grouped and averaged by doses of insensitivity alleles.

Genotypes (Lines)	FLN	Phyll I (^oC d $leaf^{-1}$)	Phyll II (^oC d $leaf^{-1}$)
A_P+B_P+D_P	11.5±0.2	84.6±06.8	133.5±08.2
A_{GS}+B_P+D_P	10.6±0.3**	86.9±05.7	130.5±09.7
A_P+**B_S**+D_P	10.4±0.4**	85.9±07.5	122.8±07.7
A_P+**B_{CS}**+D_P	10.8±0.6*	86.6±06.2	*118.7±06.5*
A_P+**B_R**+D_P	11.1±0.6 ns	85.1±06.7	*112.5±10.5*
A_P+B_P+**D_S**	10.5±0.4**	85.4±06.5	114.8±11.0
$Average_{SINGLE}$	10.7±0.1**	86.0±01.3	*119.8±01.9*
A_{GS}+B_S+D_P	10.8±0.3*	83.1±05.6	*110.5±08.6*
A_{GS}+B_{CS}+D_P	10.1±0.6***	90.3±04.7	*108.2±11.8*
A_{GS}+B_P+**D_S**	10.0±0.6***	87.0±05.9	117.3±10.8
A_P+**B_S+ D_S**	10.7±0.3*	81.8±06.0	*106.6±08.6*
A_P+**B_{CS}+D_S**	10.4±0.3**	85.5±05.8	*110.0±03.8*
$Average_{DOUBLE}$	10.4±0.2***	85.5±01.8	*110.5 ±01.3*
A_{GS}+B_S+D_S	10.2±0.8***	85.3±12.1	*110.6±16.2*
A_{GS}+B_{CS}+D_S	9.6±0.5***	91.6±05.9	*110.8±08.2*
$Average_{TRIPLE}$	9.9±0.3***	88.5±00.2	*110.7±03.4*

Values indicate means ± standard errors of the means (SEM). Values in italic type show that differences in phyllochron between NILs and wild type-Paragon were larger than their SEMs. Asterisks indicate statistical differences in FLN from LSMeans contrast against the wild type, Paragon (*$P<0.05$, **$P<0.01$, ***$P<0.001$, any symbols $0.10>P>0.05$ and ns-non significant at $P>0.10$).

The advancement of anthesis time produced by insensitivity to photoperiod was therefore independent of any effects on the phyllochron of early leaves (Fig. 3.5, middle panel), and the main cause of reducing the FLN was complemented by a reduction in phyllochron of the late leaves (Fig. 3.5, right panel).

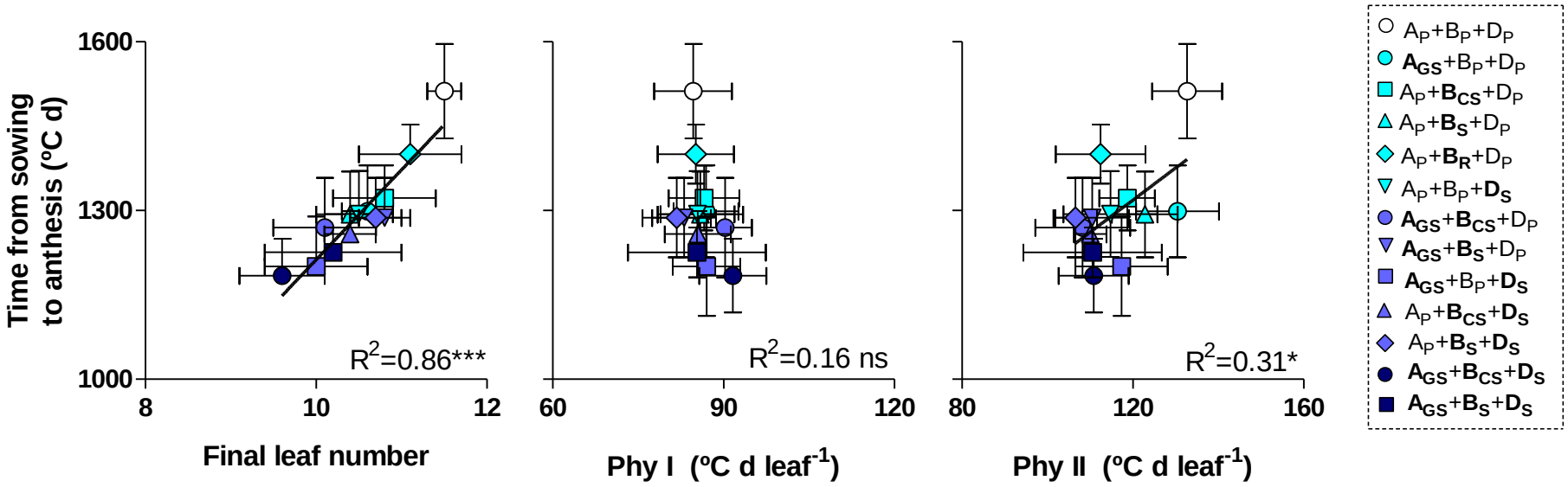

Fig. 3.5. Relationship between the duration of the period from sowing to anthesis and final leaf number (left panel), phyllochron of early (Phy I, middle panel) and late leaves (Phy II, right panel) for the different NILs and Paragon. The coefficient of determination (R^2) and the level of significance (*$P<0.05$, ***$P<0.001$, ns-non significant) were included. Segments stand for the standard error of the means.

3.3.4. Dynamics of tillering and its coordination with leaf appearance

Generally, there were not consistent effects of photoperiod insensitivity alleles on tillering dynamics (Supplementary Table TS3.1). Overall NILs there was a trend for *Ppd-1a* alleles to advance the onset of tillering, but this was not only minor but also only exceptionally significant statistically, and there was no effect on the rate on the duration of tillering dynamics. Consequently, the maximum number of tillers and the number of spike-bearing tillers at anthesis was not significantly affected (Table TS3.1). When tillering dynamics was analysed using phyllochrons (number of emerged leaves), instead of thermal time, we found no evidences of consistent and relevant effects of these alleles on the coordination between tillering and leaf appearance (Table TS3.1).

3.4. Discussion

There was a rather large effect of the growing season on the most relevant traits considered. As large effects on duration of developmental phases can be only ascribed to photoperiod and temperature (Hall *et al*., 2014; Slafer *et al*., 2015), the large differences between seasons was unexpected. Photoperiod does not change between years and as sowing times were very similar, average photoperiod for each developmental phase was identical between the two growing seasons. Therefore, the factor responsible for the variation between seasons had to be temperature. As we estimated the durations in thermal time, differences in temperatures between seasons should have been accounted for, if it is admitted that the assumptions made in the calculations were correct. We assumed, as most frequently done, universal thresholds (the same for all genotypes and all phases): a base temperature of 0ºC and an optimum temperature that would have been higher than those registered in the field, and then thermal time was simply the summation of daily average between maximum and minimum temperatures. The fact that calculated thermal time differed between seasons reveals that assumptions in calculations would not have been strictly true. As the thermal times in the warmer season were much longer than in the cooler season, it seems likely that the assumption that actual maximum temperatures would have been always lower than the optimum threshold may have been wrong. As developmental rates are reduced by temperature increases beyond the optimum threshold the warmer temperatures would have produced the delay in development. This would support the evidences from studies on the

relationships between rates of development and temperature revealing that the optimum temperature might well be relatively low (Porter and Gawith, 1999; Porter and Semenov, 2005; Slafer and Rawson, 1995b; 1995c). Notwithstanding the growing season effect, the differences between lines were highly significant and even though the interaction of the genotypes (NILs) and growing season was statistically significant; its magnitude was much smaller than the genotypic effect. This implies that the differences between lines were reasonably consistent across seasons, allowing the results be analysed averaged across of both growing seasons (as in Borràs-Gelonch *et al*., 2009, Gonzalez-Navarro *et al*., 2016).

3.4.1. Phenology

We confirmed that the introgression of *Ppd-1a* alleles accelerated the development towards flowering (Bentley *et al*.,2011,Wilhelm *et al*., 2009); with an additive effect of combining *Ppd* insensitivity alleles in different genomes (e.g. Jones *et al*., 2017; Shaw *et al*., 2012). But, well beyond this confirmation we offered novel results that shed light (i) on the complex nature of the interaction between different *Ppd-1a* alleles, and (ii) on the effects of these alleles on the components of time to anthesis both in terms of sub-phases and in terms of number of leaves initiated and rate of leaf appearance.

Regarding the complex interactions between *Ppd-1a* alleles on responses of anthesis time, they seemed to depend on the cultivar origin, on how many genomes carried photoperiod insensitivity, and on the source of the specific *Ppd-1a* allele. Concerning the particular genome in which a *Ppd-1a* allele was introgressesd, we found that, when Sonora 64 was the donor of *Ppd-B1a*, this allele and *Ppd-D1a* had the greatest effects on advancing anthesis (and *Ppd-B1a* slightly, though not significantly, stronger than *Ppd-D1a*). When considering this particular source of *Ppd-B1a*, our results would somewhat disagree with most of the literature, in which lines with *Ppd-D1a* introgressed flowered significantly earlier than those with *Ppd-A1a* or *Ppd-B1a* alleles (Bentley *et al*., 2011; Bentley *et al*., 2013; Díaz *et al*., 2012; González *et al*., 2005b; Jones *et al*., 2017; Worland *et al*., 1998). In addition, this general finding would be in line with conclusions, from a study comparing 410 European cultivars, that flowering time is mainly controlled by *Ppd-D1* and could then be exploited when relatively large adjustments in flowering time are needed, whilst *Ppd-B1* would be mainly responsible for relatively minor effects and could be exploited only for fine-tuning development to local conditions (Langer *et al*., 2014). However, the disagreement between our results

and those most commonly reported depended is partial and not unique. It is partial because if we considered the strength of *Ppd-B1a* from other sources in our results, its strength would be less than that of *Ppd-D1a*, as it is the most frequent case reported (see above references). It is not unique, as examples in which lines with *Ppd-B1a* flowered earlier than or simultaneously with lines carrying *Ppd-D1a* can be also found (e.g. Scarth and Law, 1984; Whitechurch and Slafer, 2002). Our results (as well as those from most literature) also conflict those from Stelmakh (1998) who found that *Ppd-A1a* had the strongest insensitivity effect on heading date. The inconsistency in the literature regarding the strength of *Ppd-1a* alleles depending on the particular genome is further reinforced in our own dataset when combining particular *Ppd-1a* alleles with doses: the rankings we could establish considering exclusively lines with a single dose, did not hold when these alleles were combined with other alleles effecting together time to anthesis. We therefore proved what could be inferred from the conflicts in the literature, that even though it is generally accepted that the ranking of strength of photoperiod insensitive alleles is *Ppd-D1a* > *Ppd-B1a* > *Ppd-A1a*, the actual strength of each allele would depend not only on the particular genome in which it is introgressed but also (i) on the source of the insensitive allele, (ii) on the interactions of the allele with both other *Ppd-1a* alleles, and (iii) on the genetic and environmental backgrounds in which they are evaluated. These complex interactions could be seen as an inconvenience for generating simple models of actions guiding breeders interested in manipulating time to anthesis in wheat, but it is simultaneously a source of richness in opportunities for fine-tuning adaptation of the crop. Perhaps counting with such richness is behind the extremely wide range of adaptation of wheat, making it a universal crop grown in most regions of the globe (from 60ºN to 40ºS passing through the equator and from sea level to above 3000 m above sea level) (Slafer and Satorre, 1999). We showed explicitly in the present study the relevance of the source of the *Ppd-B1a* alleles, having the weakest and strongest effect on time to anthesis when its donors were Recital and Sonora 64, respectively, and intermediate when it was Chinese Spring. Bentley *et al*. (2011) and Díaz *et al*. (2012) in experiments carried out in a photoperiod glasshouse in summer -but controlling photoperiod to be 10 h- in the UK also found a weaker effect of *Ppd-B1a* alleles from Chinese Spring than from Sonora 64, which suggest that even in such contrasting conditions (isolated plants in a glasshouse in summer of the UK in previous studies; canopy plots under field conditions with a normal autumn sowing in NE Spain in the present study) the different hold and therefore it can be postulated the effect of the

source in this case would be larger than the source x environment interaction. Then depending how fine the adaptation is to be tuned in a particular breeding programme, one or other source of *Ppd-B1a* could be more appropriately exploited. This difference in the strength of *Ppd-B1a* alleles to modify time to anthesis was likely reflecting their different polymorphism for these alleles (Díaz *et al*., 2012).

Regarding the effects of these alleles on the components phases of time to anthesis, an overall view of relationships (all linear, positive and highly significant) would suggest that these alleles affected similarly the vegetative, the early reproductive and the late reproductive phases. With this overall view, the interpretation of the particular genomes, doses and sources made for time to anthesis could be extended to each of the component phases. However, a closer inspection showed that while the duration of the vegetative and the late reproductive phases were significantly reduced by *Ppd-1a* alleles with the exception of *Ppd-B1a* from Recital, the duration of the early reproductive phase was only significantly reduced by a single dosis of insensitivity when the allele was *Ppd-A1a*, which also reduced (albeit slightly) the duration of the vegetative phase, but its effect on the late reproductive phase was only significant at $P<0.1$. Thus, in the background of Paragon at least, *Ppd-A1a* may be used to advance anthesis mainly through reducing the duration of early phases with negligible effects on the duration of the late reproductive phase. This may be relevant as the late reproductive phase is critical for determining yield (e.g. Slafer, 2003), and reducing the growth during the vegetative and early reproductive phases may not affect yield, as proven empirically by Fischer (2016). Oppositely, if the fine-tuning of anthesis date is achieved by introgressing the *Ppd-D1a* considered in the present study, all phases would be reduced but the effect on the early reproductive phase was not significant and that on the late reproductive phase was stronger than that on the vegetative phase. The strong effect of this allele on the late reproductive phase agrees with González *et al*. (2005b) who found this with different genetic backgrounds and different sources of the insensitivity allele that we used in the present study. Summarizing, the particular pattern of development partitioning through affecting more or less the durations of vegetative, early reproductive and late reproductive phases could be manipulated through the use of particular alleles for insensitivity. This in turn implies that the sensitivity to photoperiod varies through these phases and the specific strength of the sensitivity is affected by particular alleles, genomes and doses, in agreement with what had been theoretically proposed long time ago (Halloran and Pennel, 1982; Slafer and Rawson, 1994), and

with earlier findings from comparing single chromosome substitution lines (Whitechurch and Slafer, 2001).

3.4.2. Final leaf number, phyllochrons and its coordination with anthesis

Most of the effects of *Ppd-1a* alleles on time to anthesis can be seen as a consequence of the effects on FLN and duration of phyllochron, as suggested by Kirby (1990). *Ppd-1a* alleles consistently reduced FLN, as expected from previous results (Whitechurch and Slafer, 2002; González *et al*., 2005b). Even though the reduction of FLN by photoperiod insensitivity alleles was the dominant cause of responses of time to anthesis to the introgression of *Ppd-1a* alleles (through genomes, doses and sources), there was also a contribution of the effects of these alleles in reducing phyllochron of late leaves. This is because, we found that there were two rates of leaf appearance; the phyllochron of the first seven leaves was shorter than that of later leaves, as it had been documented before (Slafer and Rawson, 1997). The fact that the phyllochron of the initial leaves was unaffected by the *Ppd-1a* alleles and that of the late leaves was is in line with previous findings by González *et al*. (2005b) as well as with the effect of modifying photoperiod for a particular sensitive genotype (e.g. Slafer and Rawson, 1997). As the appearance of the late leaves mostly (though not strictly) coincides with the late reproductive phase, it was not surprising that the allele affecting most this phase, *Ppd-D1a*, was also one of the strongest effect on phyllochron of the late leaves. And *Ppd-A1a* which accelerated development mainly of the early phases had negligible effects on phyllochron. In fact, the mechanism by which an allele may modify the duration of the late reproductive phase would be its effect on phyllochron of the late leaves.

3.4.3. Dynamics of tillering and its coordination with leaf appearance

Ppd-1a alleles did reduce the maximum number of tillers but as there was a complete compensation later with the rates of tiller mortality, it was clear that in the present study these alleles did not cause major differences in the number of spikes. Although, other studies showed that *Ppd-D1a* alleles may have reduced the number of tillers at heading (Li *et al*., 2002), this is not necessarily affecting the number of spike-bearing tillers and may well not represent a conflicting result (it does agree with our results on the effects on maximum number of tillers). The positive relationship between the maximum number of tillers and tiller mortality rates determining the compensation we found are rather common in the literature (e.g. Sharma, 1995). This is because alleles increasing

the maximum number of tillers differ mainly in the number of late appearing tillers, and it has been since long recognised that late-appearing tillers do not survive to produce spikes (e.g. Davidson and Chevalier, 1990). Thus lines tillering more profusely also exhibit higher rates of tiller mortality, and not necessarily higher number of fertile tillers (e.g. Borràs-Gelonch *et al*., 2009); which is the basis for the proposition of an ideotype of wheat of limited tillering firstly (Donald, 1968) and more recently the suggestion that introgressing genes inhibiting tillering would be desirable (e.g. Kebrom and Richard, 2013 and references quoted therein).

The main conclusions we reached are:

- the magnitude of the effects of *Ppd-1a* alleles on time to anthesis, depended upon

i. the specific genome in which they were introgressed,

ii. the doses of photoperiod insensitivity alleles introgressed, and

iii.the source of the specific *Ppd-1a* allele.

For instance, when Sonora 64 was the donor of *Ppd-B1a*, this allele and *Ppd-D1a* had the greatest effects on advancing anthesis. The ranking of strength of photoperiod insensitive alleles that is generally accepted, *Ppd-D1a* > *Ppd-B1a* > *Ppd-A1a*, would depend not only on the particular genome in which it is introgressed but also on the source of the insensitive allele, and on the interactions of the allele with other *Ppd-1a* alleles.

- particular alleles might be used to manipulate the partitioning of developmental time to anthesis into component sub-phases: for instance in the background of Paragon, *Ppd-A1a* may be used to advance anthesis mainly through reducing the duration of early phases with negligible effects on the duration of the late reproductive phase.

- although photoperiod insensitivity alleles advanced anthesis mainly through reducing FLN, there was a complementary contribution by *Ppd-1a* alleles through reducing phyllochron of late leaves (whilst phyllochron of the first seven leaves was unaffected). *Ppd-1a* alleles did reduce the maximum number of tillers but there was a complete compensation with the rates of tiller mortality, therefore these alleles did not cause major differences in the final number of spike-bearing tillers (and might remove potential negative effects of tiller loss on yield)

- these conclusions may have implications for breeding such as (i) depending how fine the adaptation is to be tuned one or other source of insensitivity alleles could be

more useful, (ii) if in a program it is preferred advancing anthesis through preferentially accelerating development in a particular vegetative of reproductive phase, specific insensitivity alleles could be exploited.

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3.6. Supplementary material

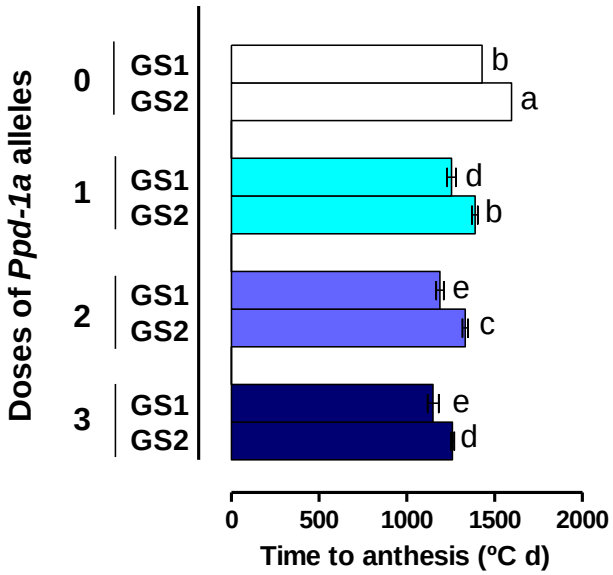

Fig. FS3.1. Duration of the period from sowing to anthesis of the different genotypes averaged across lines with the same doses of insensitive *Ppd* alleles. Different letters indicate that the differences are statistically significant ($P<0.05$). Segments in each bar stand for the standard error of the means (SEM) used to calculate the time to anthesis in the cases where different NILs were averaged.

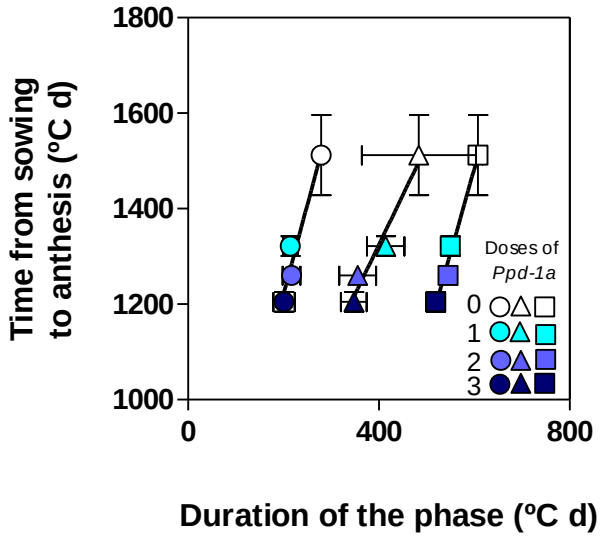

Fig. FS3.2. Relationship between time from sowing to anthesis and the duration of the three component phases (vegetative, circles; early reproductive, tringles; and late reproductive, squares) for Paragon (open symbols) and its the NILs with 1, 2 or 3 doses of *Ppd-1a* alleles (pale, intermediate and dark symbols). Durations are averaged across lines with the same doses of insensitive *Ppd* alleles.

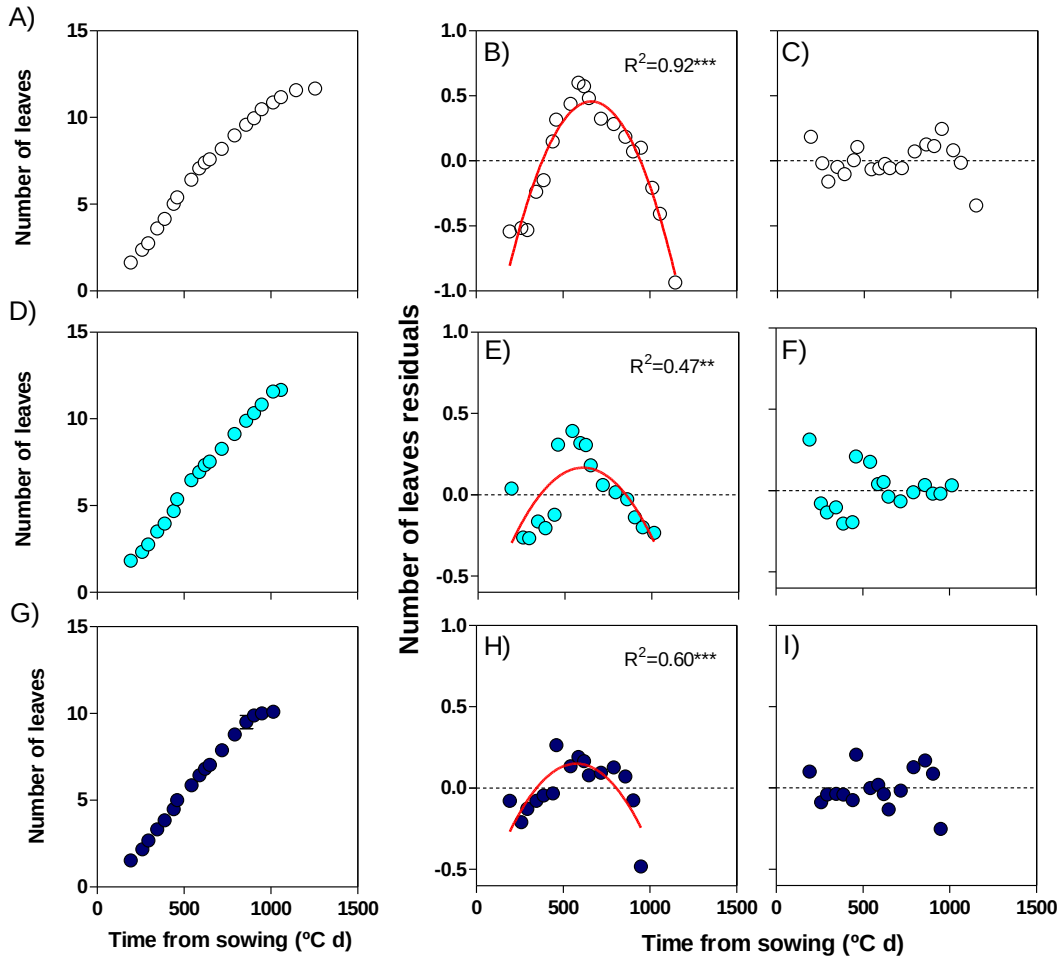

Fig. FS3.3. Relationship between the number of appeared leaves in the main shoot (Haun stage) and time after sowing (A, D, G) and distribution of the residuals of the linear (B, E, H) or bi-linear (C, F, I) regressions for these dynamics (until the appearance of the flag leaf exclusively) for the wild type Paragon (top panels: A, B, C) and the NILs with the smallest (A_P+$\mathbf{B_R}$+D_P, middle panels: D, E, F) and largest ($\mathbf{A_{GS}}$+$\mathbf{B_{CS}}$+$\mathbf{D_S}$, bottom panels: G, H, I) reductions in FLN.

Table TS3.1. Main parameters of the dynamics of tillering based on thermal time or on phyllochrons after sowing for each of the different NILs and wild type- Paragon (A_P+B_P+D_P) as well as for the averages across lines with the same doses of insensitive *Ppd* alleles. These parameters were the timing of the onset of tillering, the tiller appearance rate (TAR), the duration of tillering (TAD), the maximum number of tillers (MNT) reached at the end of tillering (as the average of the values determined from then to the onset of tiller mortality), and the final number of tillers (FNT) which are the number of fertile (spike-bearing) tillers. Tillers counted do not include the main shoot (to consider the total number of shoots, and spikes, per plant it must be added 1 to the corresponding figures).

Genotypes (Lines)	Onset of tillering (°C d)	TAR (tillers pl^{-1} [100 °C d]$^{-1}$)	TAD (°C d)	MNT (tillers $plant^{-1}$)	FNT (tillers $plant^{-1}$)	Onset of tillering (leaves)	TAR (tillers pl^{-1} $leaf^{1}$)
A_P+B_P+D_P	347.3±16.1	1.60±0.3	259.2±29.5	4.14±1.1	0.49±0.1	3.41±0.2	1.38±0.1
A_{GS}+B_P+D_P	336.5±11.5	1.55±0.2	217.3±15.8	3.40±0.8 ns	0.92±0.1 ns	3.35±0.2	1.23±0.2
A_P+**B_S**+D_P	322.2±15.9	1.59±0.3	244.9±23.8	3.87±1.1 ns	0.65±0.0 ns	3.18±0.2	1.32±0.4
A_P+**B_{CS}**+D_P	327.5±18.4	1.79±0.3	290.8±19.5	5.03±1.5 ns	0.79±0.1 ns	3.24±0.3	1.42±0.2
A_P+**B_R**+D_P	*297.8±13.4*	1.45±0.2	*338.1±40.2*	4.70±1.0 ns	1.15±0.6 ns	*2.82±0.1*	1.24±0.1
A_P+B_P+**D_S**	338.8±09.4	1.53±0.3	238.4±09.7	3.50±1.6 ns	0.53±0.1 ns	3.45±0.2	1.11±0.2
Average $_{SINGLES}$	324.6±7.3	1.58±0.1	265.9±21.7	4.10±0.3 ns	0.81±0.1 ns	3.21±0.1	1.26±0.1
A_{GS}+B_S+D_P	357.0±13.3	1.31±0.2	243.0±25.5	3.17±0.2 ns	0.81±0.1 ns	3.49±0.3	*0.94±0.1*
A_{GS}+B_{CS}+D_P	335.5±20.1	1.40±0.2	201.1±27.3	2.82±0.1 ns	0.53±0.3 ns	3.28±0.1	1.29±0.2
A_{GS}+B_P+**D_S**	*291.5±09.6*	1.33±0.2	275.7±26.0	3.33±1.2 ns	0.78±0.1 ns	3.03±0.4	*0.99±0.2*
A_P+**B_S+ D_S**	307.1±33.9	1.72±0.2	242.1±27.4	3.83±0.4 ns	1.02±0.1*	3.32±0.5	1.29±0.1
A_P+**B_{CS}+D_S**	367.3±51.7	1.37±0.5	239.2±70.7	3.24±0.2 ns	0.41±0.0 ns	3.73±0.7	1.11±0.3
Average $_{DOUBLES}$	331.7 ±14.4	1.43 ±0.1	240.2±11.8	3.28±0.2 ns	0.71±0.1 ns	3.37±0.1	*1.14±0.1*
A_{GS}+B_S+D_S	288.4±79.3	2.34±1.0	261.7±41.1	4.51±1.9 ns	0.80±0.0 ns	3.15±1.3	1.66±0.6
A_{GS}+B_{CS}+D_S	321.0±34.5	1.42±0.3	213.7±66.4	2.92±0.4 ns	0.64±0.1 ns	3.05±0.2	1.29±0.2
Average $_{TRIPLES}$	*304.7±16.3*	1.88 ±0.5	237.7±24.0	3.71±0.7 ns	0.72±0.1 ns	*3.10±0.1*	1.48±0.2

Values indicate means ± standard errors of the means (SEM), when in italic type differences between NILs and Paragon were larger than their SEMs. Asterisks indicate statistical differences in number of tillers from LSMeans contrast against the wild type, Paragon (*$P<0.05$, ns-non significant at $P>0.10$).

Chapter IV

Dynamics of leaf and spikelet primordia initiation in wheat as affected by Ppd-1a alleles under field conditions

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4. Chapter IV: Dynamics of leaf and spikelet primordia initiation in wheat as affected by Ppd-1a alleles under field conditions

4.1.Introduction

Flowering time is critical for adaptation of wheat to the many particular regions in which it is grown (Braun *et al*., 2010). Flowering time involves three major phenological phases: vegetative, early reproductive and late reproductive phases (Slafer and Rawson, 1994*a*). During these phases the organs that will become the main sources and sinks of the crop, whose balance will determine yield, are initiated as primordia. All leaf primordia, in addition to those that are already differentiated in the embryo of the seed (*c.* 4), are developed during the vegetative phase. All spikelet primordia are initiated during the early reproductive phase and all florets are produced within the spikelets during the late reproductive phase (Slafer *et al.,* 2015). The internal (apical) and microscopic processes determining leaf and spikelet primordia initiation seem to be coordinated with leaf appearance (Kirby, 1990), which in turn determines the macroscopic, external morphological stage of Haun (Haun, 1973). A major difference between these organs is that whilst all leaves and spikelets initiated will develop further and will be visible in the adult plant, within each spikelet there is an indeterminate initiation of floret primordia, most of which die (i.e. their developmental progress towards becoming fertile florets at anthesis is arrested). So the dynamics of initiation of leaves and spikelets and that of florets is dramatically different. In this paper we will focus on the dynamics of leaf and spikelet initiation and in a companion paper (Prieto *et al*., 2017) we dealt with the dynamics of floret initiation, floret mortality and the determination of fertile floret number.

Dynamics of leaf and spikelet initiation are critical in determining the final numbers of leaves and spikelets, respectively; which in turn are major determinants of yield. Final leaf number determines largely flowering time (Kirby, 1990; Hay and Kirby, 1991; Jamieson *et al.*, 1998) and therefore adaptation (Worland, 1996; Snape *et al.*, 2001), which is critical for optimising yield under particular growing conditions (Rajaram *et al.*, 1990; Reynolds *et al.,* 2012). After floral initiation all spikelets, primary branches of the inflorescence, are initiated controlling inflorescence architecture and the first component of yield through potentially affecting grains per spike (Friedman and Harder, 2004; Kellogg *et al*., 2013; Boden *et al*., 2015).

Immediately after sowing (with seed imbibition) the initiation of leaf primordia resumes. By the time of seedling emergence two additional leaves have usually been initiated. This that is why the absolute minimum final leaf number of a wheat plant is *c*. 6; (Hay and Kirby, 1991).

After seedling emergence the apex continues initiating more leaf primordia until floral initiation, when the final leaf number in the main shoot is determined (Slafer *et al*., 2015). The rate of leaf initiation is relatively constant (e.g. Kirby *et al*.,1987; Delécolle *et al.*, 1989; Miralles *et al*., 2001; González *et al*., 2002), and consequently the interval of thermal time between the initiation of two consecutive primordia (the reciprocal of the rate, termed leaf plastochron; Hay and Kirby, 1991) is constant in a particular genotype and environment for all leaves. Although it is not a fixed value, frequently leaf plastochron has been reported to be *c*. 50ºC d primordium^{-1} (Slafer and Rawson, 1994*a*).

Starting at floral initiation, all spikelets are initiated in the apex until the initiation of the last, terminal spikelet (when the number of spikelets per spike is fixed). The first spikelets might eventually be initiated at the same rate of leaf initiation but even in these cases most of the spikelets are initiated at a much faster rate (e.g. Kirby, 1974; Delécolle *et al.*, 1989 Miralles and Richards, 2000), at least when unsatisfied vernalisation requirements do not slow down development (González *et al*., 2002). As double ridge is the first (albeit microscopic) morphological sign that the apex is unequivocally reproductive (i.e. developing spikelets), this stage has been frequently considered as equivalent to floral initiation. But the change from vegetative to reproductive stage, when the first spikelet primordium is initiated, normally occurs considerably earlier (Delécolle *et al.*, 1989; Evans and Blundell, 1994). Therefore, an accurate timing of floral initiation can only be determined *a posteriori*, considering the dynamics of primordia initiation and final leaf number (as explained below in Materials and Methods).

A comprehensive understanding of how environmental and genetic factors modify these rates of primordia initiation is relevant for the design of better management and breeding strategies. Previous studies showed that leaf and spikelet initiation were influenced differently by environmental cues, such as temperature (if the rates are expressed "per day") and daylength (Delécolle *et al.*, 1989; Evans and Blundell, 1994; Miralles and Richards, 2000), and consequently respond to changes in sowing dates (Miralles *et al.*, 2001). On the other hand, very little is known of the effects of photoperiod sensitivity genes (*Ppd*) on the rates of initiation of leaf and spikelet primordia. These *Ppd* genes are major contributors to improve adaptation (Snape *et al*., 2001; Griffiths *et al*., 2009; Langer *et al*., 2014) due to their very well-known effect on flowering time. These developmental responses to photoperiod are mainly controlled by *Ppd-D1*, *Ppd-B1* and *Ppd-A1* genes (Beales *et al.*, 2007); and their effects on phenology have been quantified many times (e.g. Cockram *et al*., 2007; Fischer, 2011). But the likely effects of these genes on the rates of primordia initiation (during the

phases whose duration are affected by them) have been largely ignored, mainly because of the expertise required for, and the amount of work involved in, the proper determination of these rates (see below). In few cases, these effects were either indirectly assessed through the final number of leaves or spikelets, or studied with materials that were not isogenic lines (Scarth *et al*., 1985; Worland *et al*., 1998; Snape *et al*., 2001; Whitechurch and Slafer, 2002; Matsuyama *et al*., 2015). Finally, the very few studies that used near isogenic lines for *Ppd* genes considered a limited source of the *Ppd* alleles. And in the vast majority of cases, conclusions were taken from studies on isolated potted plants grown in controlled conditions (with severe risks when extrapolating to field canopies; Passioura, 2010; Sadras and Richards, 2014).

To the best of our knowledge, the combination of the effects of different *Ppd* genes, of sources (donors) of a particular *Ppd* allele, and of the dosage of *Ppd* genes on leaf and spikelet primordia initiation have not been analysed before. In this study, we aimed to explore under field conditions the impact of photoperiod insensitivity alleles with NILs considering different alleles, for some of them different sources, and dosage on the dynamics of primordia initiation resulting in differences in plastochron of leaves and spikelets. Furthermore, we assessed to what degree the effects of these genes on primordia initiation rates were direct or linked with their effects on other developmental processes: whether the *Ppd* effects (i) on rates of development explained part of the effects on the final number of spikelets per spike and (ii) on rates of primordia initiation were mediated through effects on the dynamics of leaf appearance (i.e. whether the effects on plastochron and phyllochron were coordinated).

4.2. Materials and methods

4.2.1. Field conditions, treatments and design

Field experiments were carried out during the 2012/13 and 2013/14 growing seasons, under stress-free conditions (i.e. weeds, insects and diseases were controlled or prevented and plots were irrigated/fertilised as required) close to Bell-lloc d´Urgell (41.63°N, 0.78°E), Catalonia, North-East Spain. The soil was classified as a complex of *Calcisol petric* and *Calcisol haplic*, following the soil classification of FAO (1990). Both experiments (2012/13 and 2013/14 growing seasons) were sown in optimum dates for the Mediterranean region of the Ebro Valley, on 24 November 2012 and 12 November 2013 respectively. We aimed to have a density of 240 plants per m^2 uniformly distributed. For that purpose, we sowed the plots at a

rate of 300 seeds m^{-2}. A week after emergence we labelled sampling areas in each plot and, when necessary, we thinned by hand these areas to have that density with very high uniformity. We counted the number of adult plants at anthesis corroborating that the plant density wanted was effectively achieved in the samples: the measured density in the sample taken at anthesis (see below) was 238±5 and 247±4 plants per m^2 in the first and second growing season, respectively.

Meteorological data were recorded daily by a meteorological station from the agro-meteorological network of Catalonia located close to the experimental site. Temperatures were warmer in the second than in first growing season, particularly the maximum temperatures during late-winter and spring, February to June, when most crop growth takes place (Table 4.1).

Table 4.1. Monthly mean minimum and maximum temperatures for the first (2012/13) and second (2013/14) growing seasons and their average for the six previous years of the experiment (from 2007 to 2012).

		Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Minimum temperature (ºC)	2012/13	3.5	-0.5	-0.8	-0.4	4.1	5.5	7.6	11.7	15.1
	2013/14	1.0	-0.8	2.6	1.7	3.3	8.6	9.0	14.0	15.5
	2007-12	2.9	0.1	0.0	0.2	2.7	6.5	10.4	13.7	16.0
Maximum temperature (ºC)	2012/13	11.8	10.7	9.9	11.8	15.8	18.8	20.4	26.9	33.8
	2013/14	10.8	7.4	11.7	12.9	17.7	22.5	24.4	29.8	30.4
	2007-12	14.1	9.6	8.8	12.9	16.7	19.8	24.8	28.7	31.2

Treatments consisted of Paragon, a spring cultivar strongly sensitive to photoperiod, with *Ppd-1b* alleles in all the three genomes (Winfield *et al.*, 2010; Shaw *et al.*, 2012), and twelve near isogenic lines (NILs) in which photoperiod insensitivity alleles (*Ppd-A1a*, *Ppd-B1a* and *Ppd-D1a*) were introgressed (alone or in combinations of two or three alleles) in its background (Fig. 4.1). NILs consisted of (i) five single NILs which carried either *Ppd-A1a*, *Ppd-B1a* (from three different donors) or *Ppd-D1a*, (ii) five double NILs with *Ppd-1a* alleles introgressed in two genomes simultaneously, and (iii) two triple NILs with *Ppd-1a* alleles on all three genomes. The sources (donors) of the introgressed insensitivity alleles were genotypes `GS-100´ (for chromosome 2A, carrying a 1027 bp promoter deletion; Wilhelm *et al*., 2009), either `Sonora64´, `Chinese Spring´ or `Recital´ (for chromosome 2B, characterised by three intact copies in tandem , one truncated copy and three intact copies in tandem, and two intact copies in tandem, respectively; Beales et al., 2007), and `Sonora64´ (for chromosome 2D, carrying a 2089 bp promoter deletion; Beales et al., 2007) (Fig. 4.1).

Photoperiod insensitivity alleles were introgressed in the background of the recurrent parent, Paragon, by crossing with the designated donors of insensitivity alleles followed by backcrossing to Paragon at least to BC_6.

Genotypes were arranged in a complete randomised design with different number of replicates (ranging from one to five replicates, depending on availability of seeds) in 2012/13. Only in two, out of the 13, genotypes grown in this first growing season the available seed was so scarce that we could only afford to sow a single field plot (and then there was only one replicate). These were A_P+**B_R**+D_P (Paragon with a single insensitive allele from Recital in chromosome 2B) and **A_{GS}**+**B_S**+**D_S** (the triple insensitive NIL with insensitive alleles from GS-100 in chromosome 2A and from Sonora 64 in chromosomes 2B and 2D). As we multiplied the seeds and produced our own stock in the first growing season, in the second experiment carried out in the 2013/14 growing season the treatments were arranged in a completely randomised block design with three replicates. Plot size was always of 6 rows (0.20 m apart) wide and 4 m long.

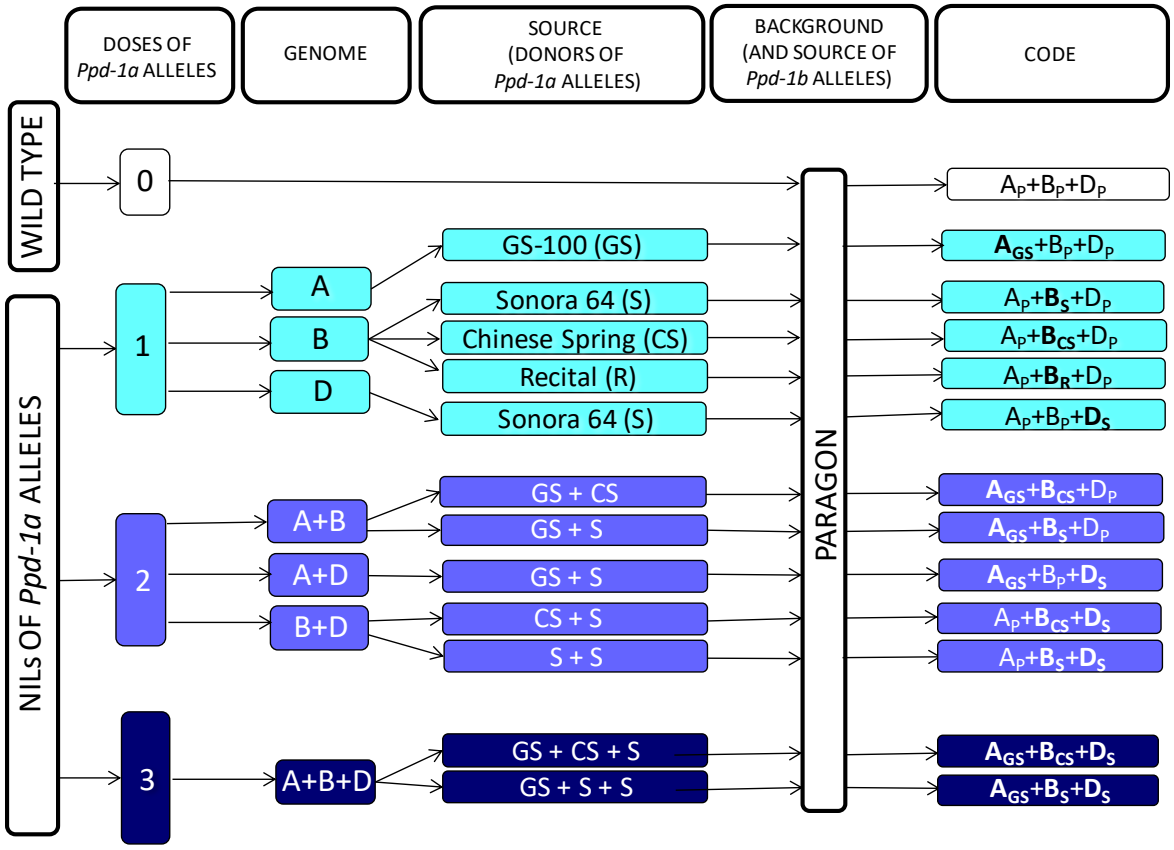

Fig. 4.1. Scheme of genotypes used in this study. Paragon, a spring wheat carrying no insensitivity alleles and its near isogenic lines (NILs) with 1, 2 or 3 doses of *Ppd-1a* alleles introgressed. The scheme indicates the genome in which photoperiod insensitive alleles were introgressed, the donors of these *Ppd-1a* alleles, and the code followed in this work for naming the different genotypes (it consists of the capital letters A, B and D representing the three genomes with a subscript indicating the source of the *Ppd-1a* alleles. The subscript `P´ indicates that the *Ppd* alleles were sensitives (*Ppd-1b* alleles) and belongs to Paragon (in plain text), while the other subscripts refer to the different

donors of *Ppd-1a* alleles (in bold type).

4.2.2 Measurements and analyses

From seedling emergence onwards, data were taken periodically for (i) leaves appeared (to use it as an alternative to thermal time as the independent variable to analyse the dynamics of primordia initiation), and (ii) cumulative number of primordia initiated in, as well as the stage of development of, the apex.

For determining leaf appearance, three plants per plot that were at the expected plant density in its surrounding area and that were representative of the cohort of emergence were selected, labelled and then monitored. In each sampling the number of leaves appeared in the main shoot (Haun, 1973) was recorded. Then the cumulative number of appeared leaves was plotted against thermal time and the reciprocal of the slope of the relationship was the phyllochron for that particular treatment. Thermal time was calculated with mean air temperature (i.e. assuming a base temperature of 0ºC and that Tmax was never above the optimum temperature).

For determining the stages of apex development and quantifying the number of primordia initiated over time, one representative plant per plot was randomly sampled at frequently intervals (from once to thrice a week, depending on temperatures). All in all there were twenty-seven plants sampled and dissected from each plot from seedling emergence to the 1-2 weeks after anthesis. In all these cases, sampled plants were taken to the lab and dissected under a binocular microscope (Leica MZ 7.5, Leica Microsystems, Heerbrugg, Switzerland). We firstly determined the developmental stage of apex (according with the scale produced by Waddington *et al*., 1983) and then counted the total number of primordia (leaf and spikelet), including the number of leaves already grown that were removed to dissect the apex (Fig. 4.2, top panels). With these data, the dynamics of leaf and spikelet primordia initiation was analysed by plotting the number of primordia against thermal time from sowing and fitting bi-linear regressions (Fig. 4.2, bottom panel). The first phase in which initiation of leaf primordia took place was much slower than the second phase when spikelet primordia were initiated. The bilinear model we fitted had a fixed intercept of 4 primordia (i.e. corresponding to the 4 leaves initiated during seed filling in the mother plant and, therefore, already developed in the embryo of the seeds) and a fixed change in slope at the timing of floral initiation of each particular genotype x experiment (i.e. when the number of primordia was 1 in excess of the final leaf number). Plastochron -thermal time elapsed between the initiation

of two successive primordia in the apex- was estimated as the reciprocal of the rate of primordia initiation. Thus, there were two distinct plastochrons estimated from the bi-linear regressions: the first corresponding to the leaves was much longer than the second which corresponds to spikelet plastochron. Timing of floral initiation was finally determined when the total number of primordia of the apex exceeded the final leaf number on the main shoot by 1 primordium (González *et al.*, 2002), reassessed using the fitted model and the FLN. Double ridge and terminal spikelet stages were determined under binocular microscope in the dissection process according to the scale developed by Waddington *et al*., 1983 (Fig. 4.2).

From the analysis of the dynamics of primordia initiation described above it would not be possible to establish whether any eventual effect of *Ppd* genes on the rates of leaf or spikelet initiation was direct or simply a reflection of the effects of these alleles on other developmental processes. We, therefore, analysed the relationship between leaf and spikelet primordia and number of emerged leaves (i.e. phyllochrons) instead of thermal time; so that effects of *Ppd* genes beyond those that may exert on phyllochron could be uncovered. Due to the constitutive difference in rates of primordia initiation of leaves and spikelets, this relationship presented a bilinear trend as well, and was fitted with a bilinear model. In this case the model used a fixed intercept of 6 primordia, because the origin in this relationship represents seedling emergence, and seedlings presented two additional leaf primordia to those present in the embryo, which were initiated between sowing and seedling emergence (Hay and Kirby, 1991).

As dissections continued after terminal spikelet for determining the developmental dynamics of individual florets (see companion paper, Prieto *et al*., 2017), the number of spikelets per spike was counted in each of these post-terminal spikelet dissections for each plot, and the final number of spikelets per spike was calculated as the average of all these values (Fig. 4.2). To validate this procedure, we also determined the number of spikelets per spike in a larger sample taken at anthesis. This sample consisted of all plants in 0.5 m long of a central row, which had been labelled after seedling emergence warranting that the plant density and uniformity was that ideally expected in the sample area and its borders. In these plants the spikelets were counted on main shoot- and tiller-spikes separately.

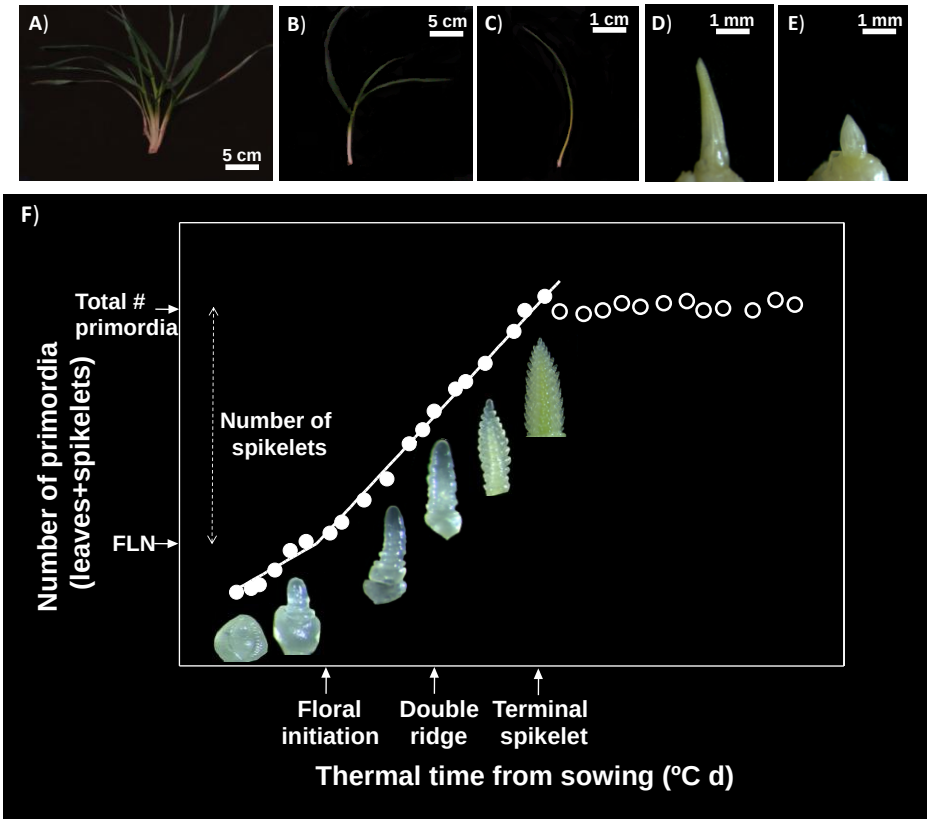

Fig. 4.2. Schematic diagram of apex dissection (top panels from A to E): expanded leaves are removed firstly (A-C) and then the unexpanded leaves covering the apex are also removed under binocular microscope (D and E). Once the apex is reached, (i) its stage is determined, according to the scale proposed by Waddington *et al*. (1983) until terminal spikelet (pictures inside bottom panel, F) and (ii) the number of primordia (leaves+spikelets) is counted. Then the number of primordia is plotted against thermal time in order to analyse the dynamics of primordia initiation as illustrated, within the period from seedling emergence and terminal spikelet (closed symbols; F). Final leaf number (FLN) is determined counting the appeared leaves following the scale of Haun (1973). Total number of primordia is the average of the values measured in a number of samples (> 10) taken from terminal spikelet to anthesis (open symbols; F). Number of spikelets is calculated as the differences between total number of primordia and FLN. Floral initiation is estimated *a posteriori* (there is no morphological evidence in the dissected apex for this critical stage) as when the first reproductive primordium was initiated; i.e. when total number of primordia exceeded by one the FLN.

4.3. Results

In order to validate our determinations made from individual plants of each plot sampled repeatedly through the growing season, we compared the number of spikelets per spike determined through the dynamics of primordia initiation with the same variable measured in the plot sample taken at anthesis. We observed that the number of spikelets per spike determined averaging the many individual plants we sampled from terminal spikelet to anthesis was the same value that we determined in the larger sample taken in anthesis (Supplementary Fig. S4.1).

This was also consistent with the fact that in all cases, there was a rather constant value of total number of primordia in the many measurements made after terminal spikelet (with small variation that can be expected as in each sampling time a spike of a different plant was quantified; see plateau in Fig. 4.3), providing confidence in the number of spikelets per spike determined at the terminal spikelet stage and quantified as the average of all dissections made from then on.

Rate of primordia production and its reciprocal (plastochron) were calculated from plotting total number of primordia against thermal time for each particular genotype and in each growing season. The relationships are illustrated for the average of all NILs with single, double or triple doses of *Ppd-1a* alleles always compared to wild type-Paragon with *Ppd-1b* alleles in all three genomes (Fig. 4.3), but the reciprocal of both slopes (leaf- and spikelet-plastochrons) are offered for each individual case (Table 4.2). Spikelets were initiated at a much higher rate than leaves (Fig. 4.3) and therefore leaf plastochron was much longer than spikelet plastochron in all cases (Table 4.2). Averaging across all genotypes and both growing seasons, the initiation of spikelet primordia was almost 67% faster than that for leaf primordia.

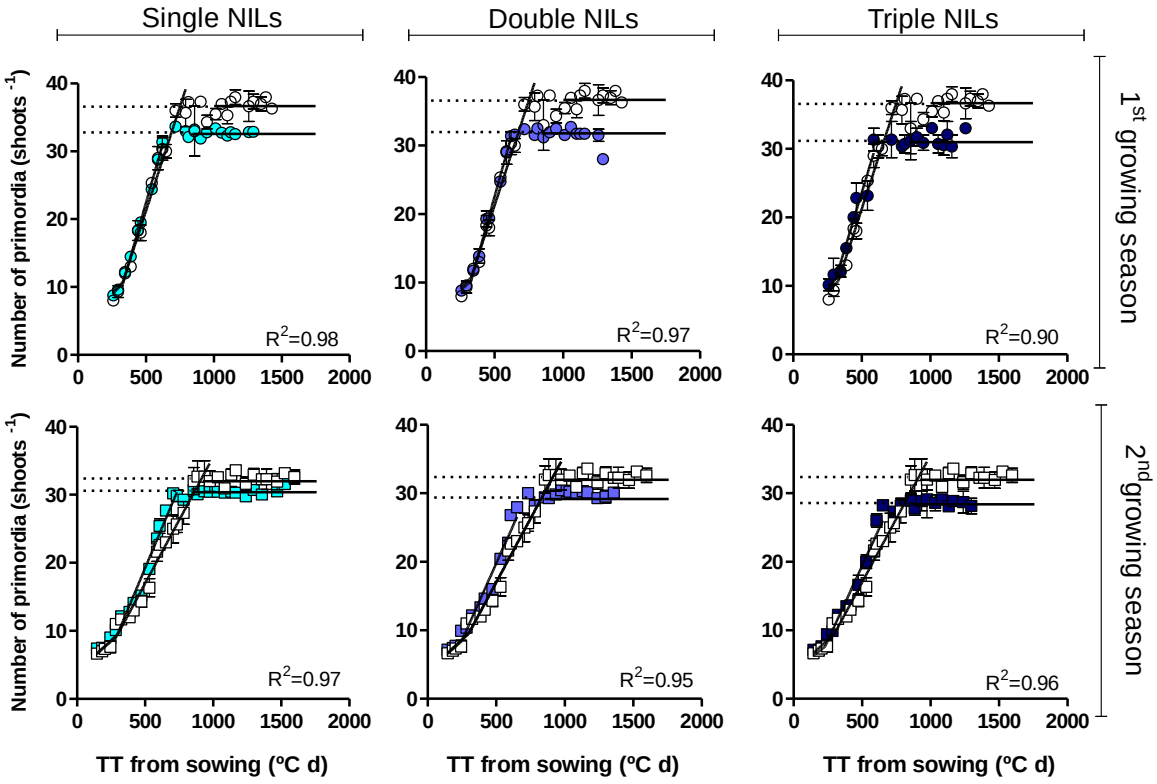

Fig. 4.3. Relationship between number of primordia (leaves+spikelets) and thermal time for NILs (closed circles) with single (light blue circles, left panels), double (blue circles, central panels) or triple (dark blue circles, right panels) doses of *Ppd-1a* alleles introgressed in the background of the wild type-Paragon (open circles) in the first (top panels) and second (bottom panels) growing seasons.

The R^2s indicated in the panels correspond to the relationships of the NILs with *Ppd-1a* alleles (R^2=0.94 and 0.95 for Paragon in the first and second growing seasons, respectively); these coefficients of determination were always highly significant (*P<0.01*).

The introgression of *Ppd-1a* alleles reduced the number of primordia initiated. In general, lines produced less primordia in the second than in the first growing season (Fig. 4.3) but in both there was a clear trend to reduce the number of primordia initiated with the increase in the doses of insensitivity alleles (Fig. 4.3). In general, data-points of the NILs having *Ppd-1a* alleles were rather overlapped with those of Paragon, although with a consistent trend to increase the rate of spikelet initiation when photoperiod insensitivity alleles were introgressed. Therefore, the main difference in the final number of primordia between lines with different doses of *Ppd-1a* alleles was explained by the timing of initiation of primordia endpoint in each case (Fig. 4.3). And the magnitude of the difference in number of primordia between NILs and Paragon was smaller in the second than in the first growing season mainly because the *Ppd-1a* alleles accelerated more clearly the rate of spikelet primordia initiation in that second season, compensating in part the advance produced in timing of terminal spikelet initiation (Fig. 4.3, Table 4.2).

Table 4.2. Leaf and spikelet plastochrons, derived from the slopes of the bi-linear relationships between number of primordia (leaves+spikelets) and thermal time (whose coefficients of determination are also included) for each of the NILs and the wild type-Paragon, A_P+B_P+D_P (as well as for the average of all NILs with the same dose of *Ppd-1a* alleles) in both growing seasons.

Genotype	First growing season			Second growing season		
	Leaf plastochron (ºC d leaf^{-1})	Spikelet plastochron (ºC d spikelet^{-1})	R^2	Leaf plastochron (ºC d leaf^{-1})	Spikelet plastochron (ºC d spikelet^{-1})	R^2
A_P+B_P+D_P	48.8±1.7	16.3±1.4	0.94	54.3±1.4	26.9±1.2	0.95
A_{GS}+ B_P+D_P	**56.0±0.6**	**13.9±0.6**	0.96	55.2±1.9	**19.9±2.0**	0.89
A_P+**B_{CS}**+D_P	46.5±1.5	14.0±1.5	0.94	**57.1±1.2**	24.2±1.5	0.94
A_P+**B_S**+D_P	**53.5±0.9**	**13.9±0.8**	0.95	53.7±1.3	**22.7±1.5**	0.93
A_P+**B_R**+D_P	47.7	16.3	0.99	**59.1±1.4**	**20.7±1.4**	0.93
A_P+B_P+**D_S**	**54.4±0.9**	**13.7±0.8**	0.96	55.6±1.2	**20.4±1.4**	0.95
$\overline{X}_{Single}$	51.6±1.9	**14.3±0.5**		56.1±0.9	**21.6±0.8**	
A_{GS}+B_{CS}+D_P	**64.5±0.8**	15.3±0.8	0.95	53.6±1.3	25.0±1.5	0.95
A_{GS}+B_P+**D_S**	46.1±1.5	13.6±1.4	0.88	**59.4±1.4**	**23.1±1.4**	0.95
A_P+**B_{CS}+D_S**	**60.2±1.0**	**13.5±1.0**	0.95	52.7±1.6	**20.9±1.8**	0.93
A_{GS}+B_S+D_P	**54.6±1.3**	14.6±1.2	0.94	**57.5±1.4**	**23.2±1.9**	0.90
A_P+**B_S+ D_S**	**59.5±0.7**	**13.9±0.6**	0.96	**57.7±1.6**	**20.0±1.7**	0.94
$\overline{X}_{Double}$	**56.8±3.3**	**14.3±0.3**		56.2±1.3	**22.4±0.9**	

A_{GS}+B_{CS}+D_S	**52.1±0.8**	**13.8±0.7**	0.93	**71.5±1.8**	**22.9±2.1**	0.90
A_{GS}+B_S+D_S	54.2	16.7	0.87	**61.2±1.3**	**22.5±1.3**	0.96
$\overline{X}_{Triple}$	**53.1±1.1**	15.3±1.5		**66.4±5.1**	**22.7±0.2**	

Values indicate mean ± standard error of the mean (SEM), with the exception of the two genotypes that were grown in a single rep in the first growing season (see Materials and Methods). Bold values indicate that differences in plastochrons between NILs and Paragon were larger than the differences between their SEMs. The R^2s were always highly significant (*P<0.001*).

When considering each individual NIL, the bilinear model fitted the data of primordia number *vs.* thermal time extremely well in both growing seasons (R^2=0.87-0.99; *P<0.01*) (Table 4.2), alike when the average of NILs having the same dose of *Ppd-1a* alleles were analysed (Fig. 4.3).

There were rather minor and inconsistent effects of *Ppd-1a* alleles on leaf plastochron while there was a significant and consistent trend for NILs with *Ppd-1a* alleles introgressed to reduce the spikelet plastochron of Paragon (Table 4.2). The effect on spikelet plastochron seemed to have been stronger for *Ppd-A1a* and *Ppd-D1a* than for the average of *Ppd-B1a* alleles. But there was variation also depending on the source of the alleles as the *Ppd-B1a* allele introgressed from Chinese Spring only marginally (and not significantly) reduced the spikelet plastochron while *Ppd-B1a* allele introgressed from Sonora 64 accelerated the rate of spikelet initiation much more strongly (and the results of introgressing *Ppd-B1a* allele from Recital was inconsistent across growing seasons; Table 4.2).

Thus, differences in the final number of primordia between different NILs and wild type Paragon were related to the effects of the photoperiod insensitivity alleles on reducing the duration of the vegetative and early developmental phases, although the relationship was significant only in the second growing season (Fig. 4.4); whilst there was no relationship whatsoever between final leaf number and leaf plastochron (Fig. S4.2, top panels) or between spikelets per spike and spikelet plastochron (Fig. S4.2, bottom panels).

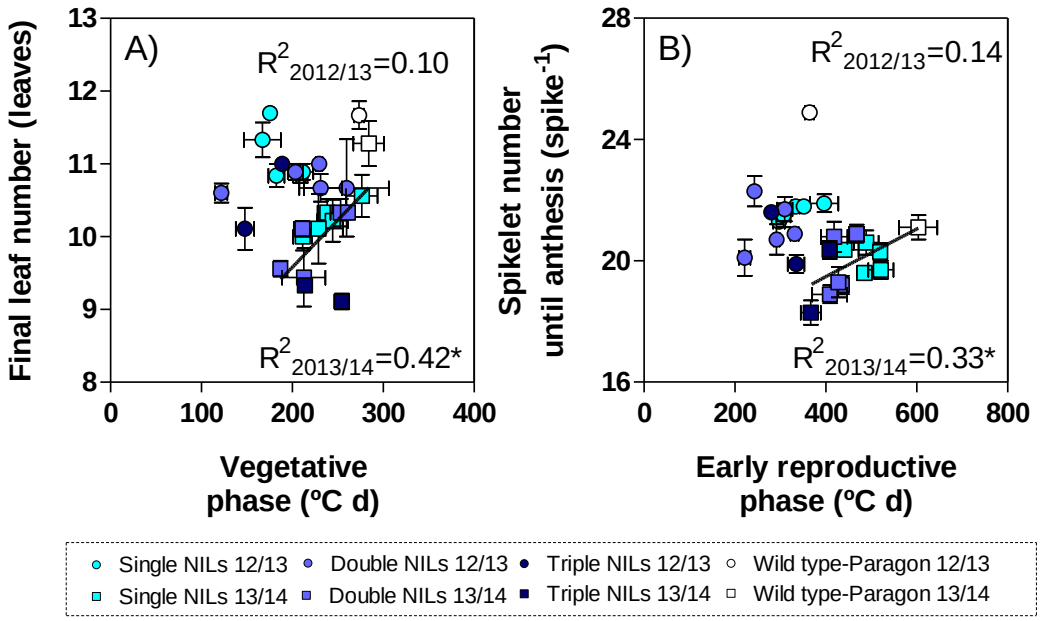

Fig. 4.4 Relationship between final number of leaves on the main shoot (A) or spikelets in the main-shoot spike (B) and the duration of the phases when these organs were initiated (vegetative and early reproductive phases, respectively) for NILs (closed symbols) with single (light blue), double (blue) or triple (dark blue) doses of *Ppd-1a* alleles introgressed in the background of the wild type-Paragon, A_P+B_P+D_P (open symbols), in the first (circles) and second (squares) growing seasons.

It is worth noting that in the second growing season (when the relationships were significant) the change in number of leaves initiated from seedling emergence (before then plants do not respond to photoperiod) to floral initiation was proportional to the reduction in duration of the vegetative phase; whilst it was not in the case for the number of spikelets initiated during the early reproductive phase and the duration of this phase. If we assume that at the time of seedling emergence seedlings had already 6 leaf primordia initiated, the number of leaf primordia initiated from then to floral initiation ranged from *c.* 3 (in the triple insensitive NILs) to 5 in Paragon, whilst the duration of the vegetative phase ranged from less than 200 to *c.* 300°C d (Fig. 4.4A). The number of spikelets per spike, on the other hand, ranged from *c.* 18 (in one of the triple insensitive NILs) to *c.* 21 in Paragon, whilst the duration of the early reproductive phase ranged from less than 400 to more than 600°C d (Fig. 4.4B). The reason for this was the differential effect of photoperiod insensitivity alleles on leaf- and spikelet-plastochron (the latter was reduced, partially compensating for the reduction these alleles produced in the early reproductive phase).

The strength of the reduction in the number of primordia in main shoots depended partly on the doses of *Ppd-1a* alleles introgressed and partly on the specific allele considered. There seemed to be a general trend for NILs with higher doses of insensitivity alleles to reduce the

duration and the final number of primordia initiated in that period, but with variation depending upon the specific allele being considered (Fig. 4.4). The genotype with the lowest leaf and spikelet numbers was one of the NILs with triple introgression (**A_{GS}+B_{CS}+D_S**) in both growing seasons (10.1±0.3 and 9.1±0.1 leaves and 19.9±0.3 and 18.3±0.4 spikelets per spike for first and second growing seasons, respectively), whilst the genotype with the highest numbers was the wild type Paragon (11.7±0.2 and 11.3±0.3 leaves and 24.9±0.2 and 21.1±0.4 spikelets per spike for first and second growing seasons, respectively).

The primordia production rate was calculated in phyllochrons as well, based on the bilinear regression fitted to the data-points of the relationships between the cumulative number of primordia initiated in the apex and the number of emerged leaves on the main shoots (Table S4.1), as illustrated for the average of all NILs with single, double or triple doses of *Ppd-1a* alleles *vs* Paragon (Fig. 4.5). On average, 1.7 leaves and 5.6 spikelets were initiated per phyllochron. Photoperiod insensitivity alleles did not clearly affect the coordination between primordia initiation and leaf emergence, but spikelet plastochron (in terms of spikelets initiated per phyllochron) tended to be slower in NILs carrying these insensitivity alleles than in wild type-Paragon (Table S4.1). In other words, *Ppd-1a* alelles reduced spikelet plastochron when calculated in thermal time, but the opposite was true when spikelet plastochron was estimated in phyllochrons. Consequently, there was not a consistent effect of the doses of *Ppd-1a* alleles on spikelet plastochron measured in phyllochrons, i.e. as doses increased not always spikelet plastochron increased. In addition, no clear effects of the source of *Ppd-1a* alleles were found on spikelet plastochron (Table S4.1).

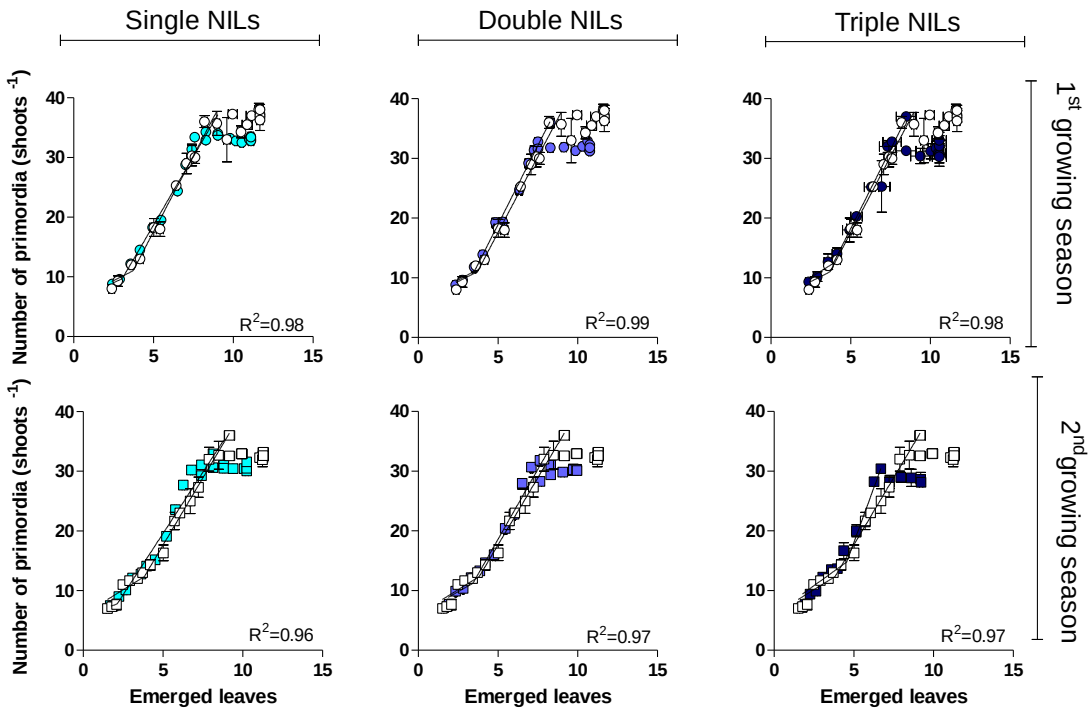

Fig. 4.5. Relationship between the number of primordia (leaves+spikelets) and the number of emerged leaves for different NILs (closed circles) with single (light blue circles, left panels), double (blue circles, central panels) or triple (dark blue circles, right panels) doses of *Ppd-1a* alleles introgressed in the background of the wild type-Paragon (open circles) in the first (top panels) and second (bottom panels) growing seasons. The R^2s indicated in the panels correspond to the relationships of the NILs with *Ppd-1a* alleles (R^2=0.985 and 0.989 for Paragon in the first and second growing seasons, respectively); these coefficients of determination were always highly significant (*P<0.01*).

Double ridge occurred later than floral initiation in all genotypes in both seasons (Fig. 4.6, with all data-points clearly above the 1:1 ratio). *Ppd* insensitivity alleles reduced the period from sowing to double ridge stage and the general trend was a stronger reduction with increasing doses of insensitivity alleles. Thus, when averaged across NILs of the same number of *Ppd-1a* alleles, NILs with single, double and triple doses of insensitivity alleles reduced time to double ridge by 10.5, 13.5 and 19.5% respect to Paragon. There was a positive trend (significant only to a probability lower than 5%) between duration of time from sowing to double ridge and that to floral initiation, indicating that the advance in development produced during the vegetative phase by the introgression of insensitivity alleles was maintained through the early reproductive phase until the appearance of the double ridge. However, the effects of *Ppd-1a* alleles on the specific gap between true floral initiation and the appearance of a double ridge in the apex were not consistent. The lack of consistency was not only revealed by the relatively low coefficients of determination but also by the fact that in the first growing season the relationship tended to converge with the 1:1 line, while in the second season the trend was to slightly diverge (Fig. 4.6). In other words, there was not a

consistent trend for insensitive lines to exhibit a more coincident timing of floral initiation and double ridge than the sensitive lines.

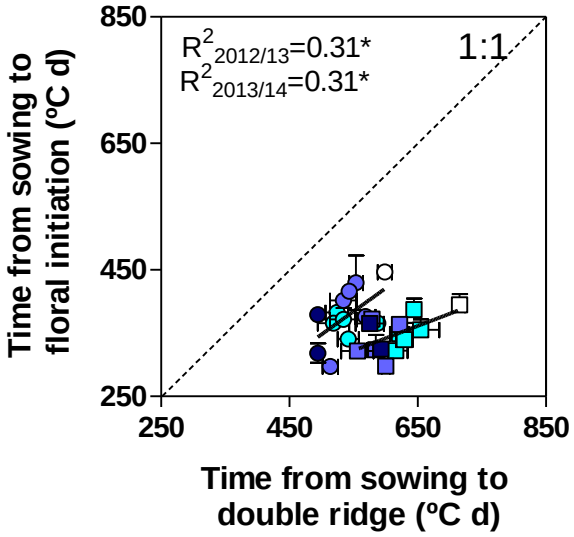

Fig. 4.6. Relationship between periods from sowing to double ridge and to floral initiation for NILs (closed symbols) with single (light blue), double (blue) or triple (dark blue) doses of *Ppd-1a* alleles introgressed in the background of the wild type-Paragon (open symbols), in both the first (circles) and second (squares) growing seasons. The dotted line stands for the 1:1 ratio, where data-points should be if double ridge was actually floral initiation.

4.4. Discussion

While *Ppd-1a* alleles accelerated the rate of phasic development during the vegetative period of leaf initiation, they did not affect the rate of leaf initiation. This finding agrees with previous studies in which leaf plastochron was not affected by the action of *Ppd-1a* alleles (e.g. Snape *et al*., 2001; Whitechurch and Slafer, 2002). Consequently, photoperiod insensitivity alleles affected FLN in parallel with their effects on the duration of the vegetative phase (e.g. Miglietta, 1989; Slafer and Rawson, 1994*b*), determined either in thermal time or in phyllochrons; mainly because phyllochron of the first emerged leaves, those that appear during the vegetative phase, are insensitive to photoperiod (Slafer and Rawson, 1997) and unaffected by photoperiod insensitivity genes (González *et al*., 2005; Ochagavía *et al*., 2017). This parallelism is the main reason why the effects of daylength and/or photoperiod insensitivity genes on wheat development can frequently be estimated through their effects on FLN (e.g. Brooking *et al.*, 1995; Slafer and Rawson, 1995; Brooking and Jamieson, 2002; Whitechurch and Slafer, 2002; González *et al*., 2005).

Spikelets were generated at a faster rate than the leaves, as it has been reported in the literature repeatedly (e.g. Kirby, 1974; Delécolle *et al*., 1989; Kirby, 1990; Miralles and

Richards, 2000) . Unlike what we described for the dynamics of leaf primordia initiation, *Ppd-1a* alleles accelerated the spikelet initiation rate (reducing spikelet plastochron) with respect to the photoperiod sensitive variety Paragon (an analogous effect to that of winter wheats; González *et al*., 2002). This increased rates of spikelet initiation produced a partial trade-off with the effects of these alleles on the duration of the spikelet initiation phase. The consequence of this partial trade-off was a relatively smaller reduction in number of spikelets per spike due to the introgression of alleles conferring photoperiod insensitivity. This genetic effect is in line with a slight increase in spikelet initiation rates observed when plants of a sensitive cultivar are grown under longer photoperiods, which partially compensates for the effects on reducing the duration of the early reproductive phase (see discussion in Slafer and Rawson, 1994*b*). In the present study, *Ppd* insensitivity alleles reduced the number of spikelets per spike (as it was frequently found elsewhere, e.g. Snape *et al*., 2001; González *et al*., 2005), but the magnitude of the reduction was not commensurate with the reduction in duration of the early reproductive phase. This is relevant as advancing time to anthesis through these genes would bring about a proportionally smaller reduction in spikelets per spike and therefore adaptation would be improved with only marginal potential losses in this yield component. *Ppd-D1a* and *Ppd-A1a* had the fastest spikelet initiation rates (the shortest spikelet-plastochrons), whilst *Ppd-B1a* alleles presented a rate of spikelet initiation slower than those of the lines with the other insensitivity alleles (though still faster than the control Paragon). It is worth noting that the proposed functional polymorphisms for *Ppd-D1a* and *Ppd-A1a* both involve a promoter deletion, with both deletions overlapping (Wilhelm *et al.*, 2009), whereas *Ppd-B1a* insensitivity is probably due to increased copy number (Díaz *et al.*, 2012). This ranking in strength for these *Ppd-1a* alleles agrees with the conclusions of Scarth *et al.* (1985) and González *et al.* (2005). In fact, the effect of *Ppd-B1a* alleles on spikelet plastochron did actually vary with the source of the allele introgressed in the background of Paragon. This might explain the controversy in the literature when in some cases it was reported that *Ppd-B1a* alleles increased rate of spikelet initiation under short photoperiod more than *Ppd-D1a* (Whitechurch and Slafer, 2002).

When plastochrons were quantified based on another developmental trait such as phyllochron, the effects of *Ppd-1a* alleles on spikelet plastochron calculated in thermal time disappeared. It means that the effects of these alleles on the rate of spikelet initiation actually reflected those on the rate of leaf appearance (Ochagavía *et al*., 2017) reinforcing the idea that the coordination between primordia initiation and leaf appearance is relatively conservative (Kirby, 1990; Miralles *et al.*, 2001). In fact, the model proposed by Kirby (1990)

for the coordination between primordia initiation and the dynamics of leaf appearance predicted that spikelet plastochron was reduced (and the rate of spikelet initiation was increased) with reductions in FLN, and our results agree with that prediction as the introgression of *Ppd-1a* alleles reduced FLN (see discussion above).

Ppd insensitivity alleles advanced the timing of double ridge (DR), as they accelerated the rates of pre-anthesis phasic development. But the fact that double ridge occurred well after floral initiation (as recognised several times in the past, Delécolle *et al*., 1989; Kirby, 1990) was not eliminated by the introgression of these alleles. The introgression of *Ppd-1a* alleles did not consistently affect the gap between true floral initiation and double ridge, indicating that differences on early reproductive phase (from floral initiation to terminal spikelet; Ochagavía *et al*., 2017) caused by these alleles were not mediated by differences in the period between floral initiation and double ridge.

We concluded that *Ppd-1a* alleles
(i) did not affect leaf initiation rate and therefore reduced FLN in parallel with reductions imposed on duration of the vegetative phase, but
(ii) accelerated the spikelet initiation rate partially compensating for their effect of shortening the length of early reproductive phase. This caused a decrease in the number of spikelets per spike that was less than proportional to the reduction in duration of the phase. The magnitude of the effects depended not only on the doses and genome involved but also on the source of a particular allele considered (in this study *Ppd-B1a*); which may well be the cause for the conflict in the literature when rankings of strengths are produced from different materials and opens potential for selecting the best homoeoallelic combinations of *Ppd-1* for reducing or delaying time to anthesis (depending on the adaptive target), while maximising spikelet number.

4.5. References

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4.6. Supplementary material

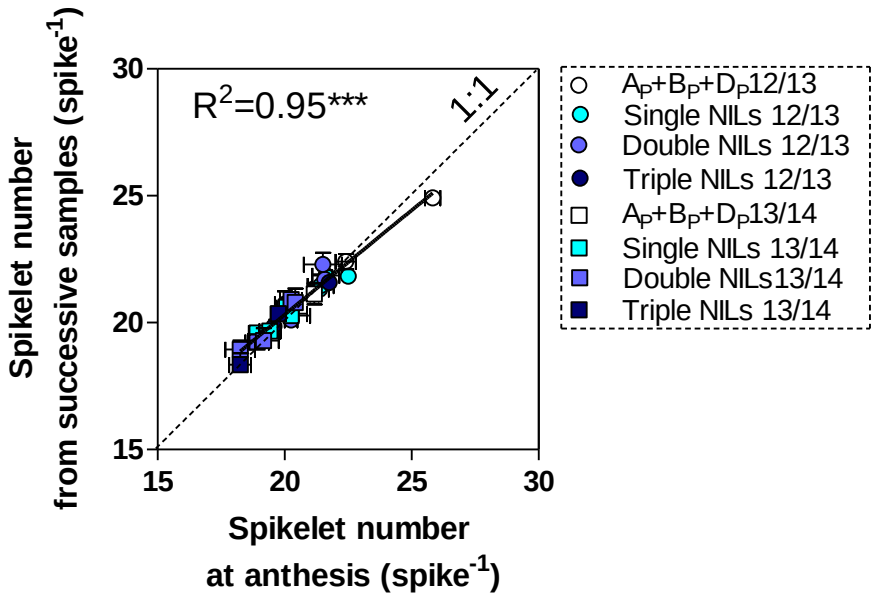


Fig. S4.1. Relationship between the number of spikelet primordia determined as the average from successive samples of individual plants taken from terminal spikelet to anthesis and that counted simultaneously in many plants from a larger sample taken at anthesis for NILs with single, double or triple doses of *Ppd-1a* alleles introgressed in the background of the wild type-Paragon, A_P+B_P+D_P in both growing seasons.

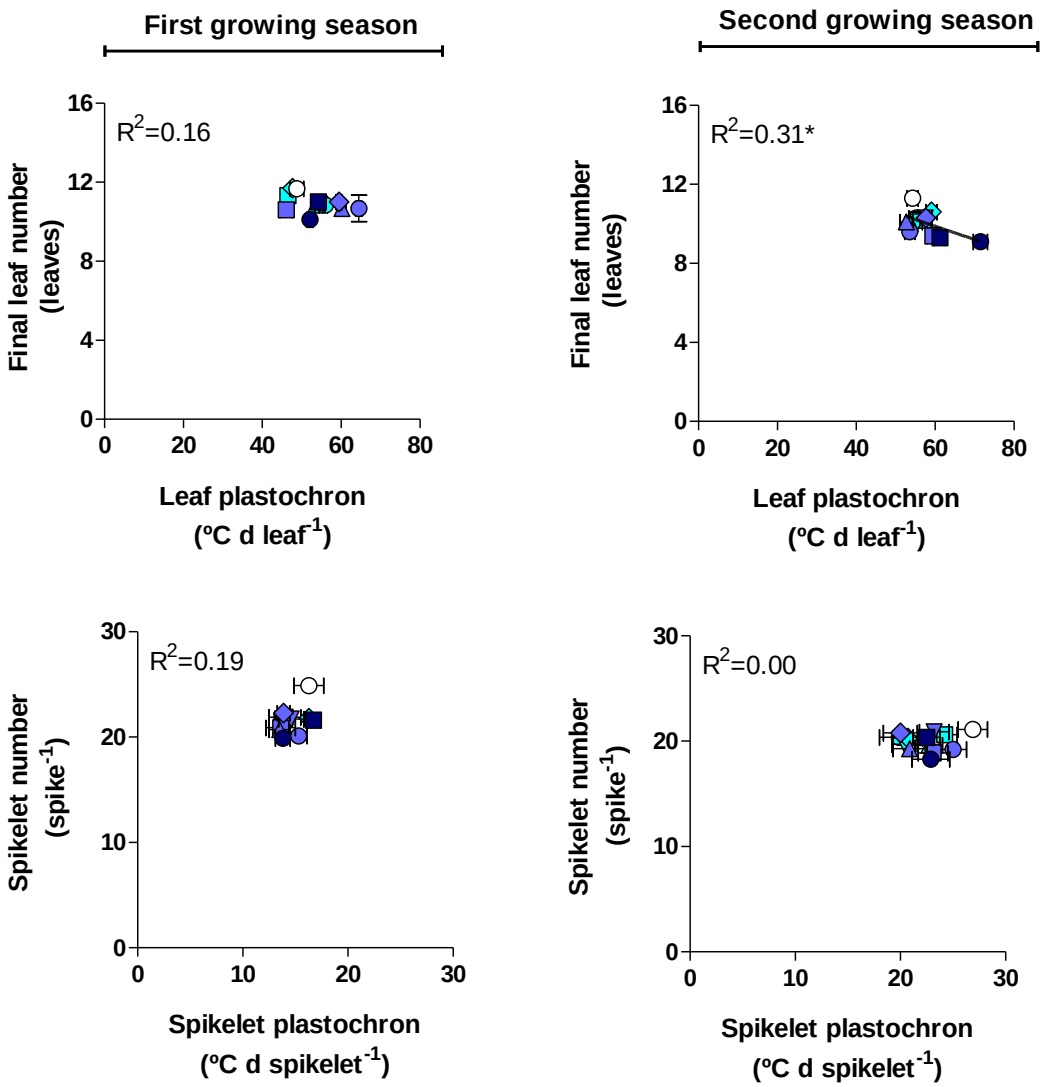


Fig. S4.2. Relationship between leaf plastochron and final leaf number (top panels) and between spikelet plastochron and spikelet number (bottom panels) for NILs with single, double or triple doses of *Ppd-1a* alleles introgressed in the background of the wild type-Paragon (A_P+B_P+D_P) in both growing seasons.

Table S4.1. Leaf and spikelet plastochrons, derived from the slopes of the bi-linear relationships between number of primordia (leaves+spikelets) and the number of emerged leaves (whose coefficients of determination are also included) for each of the NILs and the wild type-Paragon, $A_P+B_P+D_P$ (as well as for the average o all NILs with the same dose of *Ppd-1a* alleles) in both growing seasons.

	First growing season			Second growing season		
	Leaf primordia (leaf emerged^{-1})	Spikelet primordia (leaf emerged^{-1})	R^2	Leaf primordia (leaf emerged^{-1})	Spikelet primordia (leaf emerged^{-1})	R^2
$A_P+B_P+D_P$	1.35±0.27	5.09±0.31		1.66±0.16	4.50±0.24	
$\mathbf{A_{GS}}+B_P+D_P$	1.27±0.42	5.10±0.35	0.98	1.68±0.30	**5.59±0.53**	0.97
$A_P+\mathbf{B_{CS}}+D_P$	1.11±0.65	4.53±0.33	0.97	1.64±0.25	5.16±0.42	0.98
$A_P+\mathbf{B_S}+D_P$	1.54±0.25	5.60±0.53	0.98	1.85±0.21	**5.60±0.63**	0.97
$A_P+\mathbf{B_R}+D_P$	2.09	6.09	0.97	1.53±0.44	4.38±0.46	0.95
$A_P+B_P+\mathbf{D_S}$	1.65±0.20	5.70±0.50	0.98	**0.95±0.48**	4.57±0.29	0.97
$\overline{X}_{Single}$	1.53±0.17	5.40±0.27		1.53±0.15	**5.06±0.25**	
$\mathbf{A_{GS}}+\mathbf{B_{CS}}+D_P$	**2.14±0.20**	7.26±2.02	0.97	1.90±0.12	**6.15±0.95**	0.98
$\mathbf{A_{GS}}+B_P+\mathbf{D_S}$	1.23±0.63	4.89±0.42	0.97	1.90±0.16	**6.59±1.21**	0.97
$A_P+\mathbf{B_{CS}}+\mathbf{D_S}$	1.17±0.37	5.09±0.27	0.99	1.59±0.18	**5.43±0.40**	0.98
$\mathbf{A_{GS}}+\mathbf{B_S}+D_P$	**2.26±0.26**	**7.19±1.73**	0.95	1.43±0.34	4.67±0.43	0.96
$A_P+\mathbf{B_S}+\mathbf{D_S}$	1.41±0.20	**6.72±0.60**	0.98	1.84±0.22	**6.05±0.76**	0.97
$\overline{X}_{Double}$	1.64±0.23	**6.23±0.52**		1.73±0.09	**5.78±0.33**	
$\mathbf{A_{GS}}+\mathbf{B_{CS}}+\mathbf{D_S}$	1.35±0.24	5.58±0.19	0.99	1.86±0.17	**7.61±1.23**	0.97
$\mathbf{A_{GS}}+\mathbf{B_S}+\mathbf{D_S}$	1.75	5.15	0.88	1.99±0.20	**6.96±1.56**	0.96
$\overline{X}_{Triple}$	1.55±0.20	5.37±0.21		**1.92±0.06**	**7.29±0.32**	

Values indicate mean±standard error of the mean (SEM), with the exception of the two genotypes that were grown in a single rep in the first growing season (see Materials and Methods). Bold values indicate that differences in plastochrons between NILs and Paragon were larger than the differences between their SEMs. The R^2s were always highly significant ($P<0.01$).

Chapter V

Earliness per se effects on phasic development and on leaf and spikelet initiation under field conditions

5. Chapter V: *Earliness per se* effects on phasic development and on leaf and spikelet initiation under field conditions

5.1. Introduction

Flowering time is essential for wheat adaptation to wide agronomic environments (Evans, 1993; Griffiths *et al*., 2009; Hall *et al*., 2014; Kamran *et al*., 2014; Langer *et al*., 2014), and therefore it influences strongly on yield determination (Slafer, 2003; Reynolds *et al*., 2012). It can be analysed in terms of either (i) the duration of three different pre-anthesis phenological phases or (ii) final leaf number and phyllochron (complemented by the time elapsed between flag leaf emergence and anthesis). Regarding the first approach, the period from seedling emergence to anthesis is composed by three developmental sub-phases: (i) vegetative (until floral initiation), when the plant apex initiates leaf primordia, (ii) early reproductive (from floral initiation to terminal spikelet), when all spikelets are differentiated and (iii) late reproductive (from terminal spikelet to anthesis), when floret development occurs (Slafer and Rawson, 1994). Regarding the second approach, as all initiated leaf primordia must appear before the spike may emerge from the sheath of the flag leaf, the number of leaves initiated in the main shoot during the vegetative phase (final leaf number; FLN) establishes a threshold, modified by the rate at which they appear, for the time to anthesis. Both approaches are actually more complementary than conflicting (Slafer *et al*., 2015). This is because the duration of the vegetative phase may affect FLN (depending on whether the factor affecting this duration does or does not affect the leaf plastochron), and both the number of leaves that must appear during reproductive phases and the value of phyllochron may be the cause for difference in duration of these phases.

Generally, the effects of developmental genes in wheat have been mostly explored considering time to heading or anthesis as a unique phase. Although, due to the simplicity of scoring heading/anthesis, this has the advantage that allows analysing developmental differences in large populations affordably, it has the drawback that the effects of genes on duration of particular phases (as well as on the initiation of organs that take place then) are overlooked. To quantify these effects is worthwhile if we are interested not only on the effects of these alleles on adaptation (chiefly governed by time to anthesis) but also in yielding capacity. This is because during the different phases different organs are being initiated and changes in crop growth during these phases affect yield differently (e.g. Fischer, 1985; Savin and Slafer, 1991; Abbate *et al*., 1997; Demotes-Mainard and Jeuffroy, 2004; González *et al*., 2005*a*; Fischer, 2011; Ferrante *et al*., 2012; Fischer, 2016). Therefore, genetic manipulation

of the specific durations of vegetative, early reproductive and late reproductive phases could be critical for improving simultaneously adaptation and yielding capacity (Slafer *et al*., 2001; Miralles and Slafer, 2007). This sort of genetic improvement would be more certain if we could disclose and quantify the effects of particular alleles on specific phases composing time to anthesis. The other physiological approach to understand the effects of particular genes on time to anthesis (mainly final leaf number and phyllochron) is also relevant. The relevance is based on the fact that dynamics of leaf appearance is critical for determining early vigour and the rate of tillering (Kirby *et al*., 1985) which may, in turn, contribute to the determination of the final number of spikes of the crop, a key component of yield. Although coordinated, the specific parameters of the relationship between tillering and leaf appearance may be variable (Alzueta *et al*., 2012). Identifying genes affecting phyllochron should be complemented with quantifying their effects on tillering dynamics.

Genetic variation in flowering time is mainly controlled by diverse alleles of three groups of genes. Two of them are explicitly recognised to interact with the environment, controlling responses to photoperiod (*Ppd*) and vernalization (*Vrn*). The third group is responsible of relatively smaller variations, but that may be critical to provide a fine-tuning for adaptation (Griffiths *et al*., 2009) and become evident when identifying genotypic differences in phenology once vernalization and photoperiod requirements were totally satisfied (Appendino and Slafer, 2003). This group is integrated by a number of earliness *per se* (*Eps*) genes (Worland *et al*., 1994), known to provide a certain intrinsic earliness (Masle *et al*., 1989) determining a basic vegetative phase duration (Major, 1980; Flood and Halloran, 1984) as discussed by Slafer (1996). The effects of *Eps* genes on flowering time are far less known than those of alleles of the other two groups. In part, this may be due to the fact that there are too many different *Eps* genes (to the level that they may be in all chromosomes of wheat; Kamran *et al*., 2014; Lopes *et al*., 2015), that in many experimental approaches can be masked by the presence of *Vrn* and *Ppd* genes (Zikhali *et al*., 2014; Sukumaran *et al*., 2016). Although in most studies, the effects of *Eps* were measured only on time to heading or anthesis, it seemed likely that the effects might be different in vegetative and reproductive phases (Slafer, 1996). For instance, Lewis *et al*. (2008) determined that the duration of both the vegetative and early reproductive phases was affected by *Eps* genes, causing also changes in the number of spikelets per spike (an effect also reported by Alvarez *et al*., 2016). But experiments reporting effects of *Eps* genes on particular phases and on phyllochron and tillering dynamics are very scarce and limited to very few of the many *Eps* genes identified so far and, to the best of our knowledge, there are no studies on the effects of these alleles on the

dynamics of leaf and spikelet initiation in the apex. This is critical to determine whether or not the effects of particular *Eps* genes on duration of vegetative and reproductive phases produce a trade-off in the number of organs being initiated. Griffiths *et al*. (2009) found significant earliness *per se* QTLs analysing heading time in four double haploid populations of wheat developed by crosses between UK adapted parents. They were located in chromosomes 1DL and 3A. These two QTLs were identified in populations of both Avalon x Cadenza (AxC) and Spark x Rialto (SxR). Then, near isogenic lines (NILs) were developed in order to validate these important QTLs and understand its expression. Zikhali *et al*. (2014) studied four independent NILs pairs segregating for the 1DL QTL from SxR. They showed that NILs with the *Eps* allele of Spark flowered 3-5 days earlier than NILs with the Rialto allele. Farré *et al*. (2016) used several NILs for different QTL regions, developed from AxC exploring the effects on diverse physiological traits. We took a step forward evaluating the *Eps* alleles effects using different NILs for these 1D and 3A *Eps* QTLs under field conditions on adaptation and yielding building capacity traits, such as duration of different phases simultaneously with the dynamics of leaf and spikelet initiation and those of leaf and tiller appearance, developmental traits that were never studied comprehensively (and even less under field conditions).

5.2. Material and methods

5.2.1. General descriptions

Three field experiments were carried out during 2012/13, 2013/14 and 2014/15 growing seasons in two locations of Lleida, NE Spain (for the 2012/13 and 2013/14 growing seasons the experiments were located close to Algerri: 41.8ºN, 0.64ºE; while the third experiment was carried out in 2014/15 close to Bell-lloc d'Urgell: 41.6ºN, 0.77ºE). Experimental plots were sown on 2 November 2012, 7 November 2013 and 14 November 2014, in all cases at a rate of 300 seeds m^{-2}. Soil types were classified as *Fluvisol calcari* and *Calcisol petric* (in 2012/13 and 2013/14) and as a complex of *Calcisol petric* and *Calcisol haplic* (in 2014/15) following the soil classification of FAO (1990). Supplementary irrigation was applied as needed to avoid water stress (equivalent to 90, 190 and 240 mm, in the first, second and third growing season, respectively). Monthly accumulated rainfall, maximum, minimum and mean temperatures of these experiments were shown in Fig. 5.1. Plants were naturally vernalised in the field during 114, 102 and 90 days (i.e. days with mean temperatures between 0 and 10 °C and whose maximum temperatures did not exceeded 15 °C) in the first, second and third

growing seasons, respectively. Photoperiod at each day after seedling emergence were virtually the same across growing seasons (minimum differences due to differences in sowing dates), and ranged from 8.97 to 15.03 h, including and civil twilight (see Table 5.1).

Table 5.1. Photoperiod, including civil twilight, for particular timings (starting of the crop perception of daylength, and two critical stages of development considered in this study; as well as for the winter and summer solstices) throughout the growing seasons (2012/13, 2013/14 and 2014/15). For these calculations we used the average date of each of the three stages across the population.

	Seedling emergence	21st Dec	Terminal spikelet	Anthesis	21st June
	---------- h:min ----------				
1st growing season	10:42	10:12	12:55	15:45	16:21
2nd growing season	10:38	10:12	13:27	15:29	16:21
3rd growing season	11:27	10:13	13:34	15:21	16:20

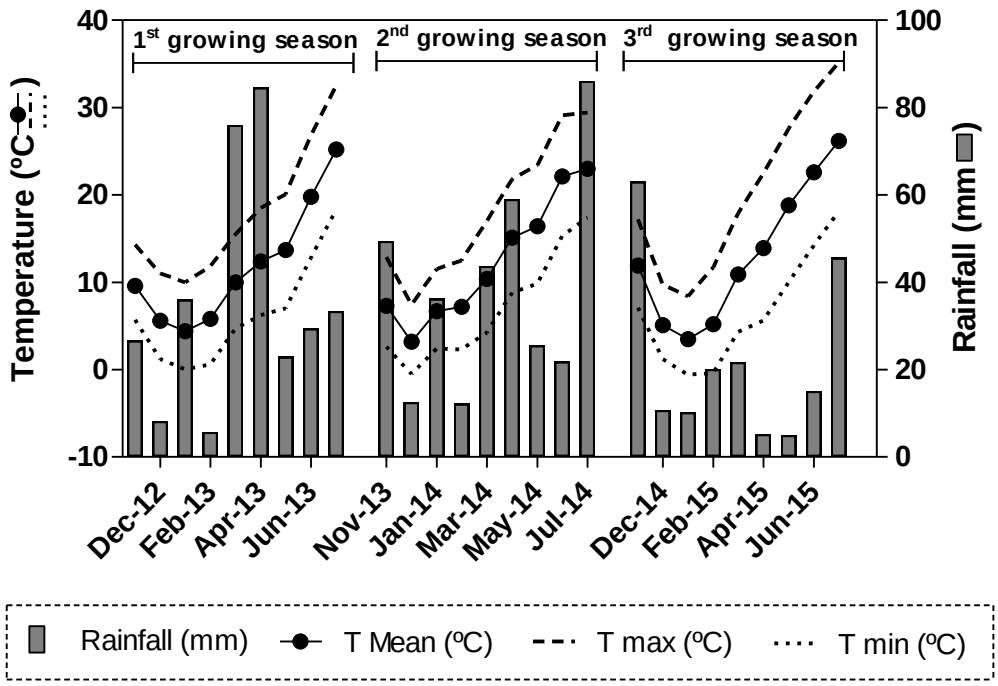


Fig. 5.1. Monthly accumulated rainfall, and mean, maximum and minimum temperatures during the three growing seasons (2012/13, 2013/14 and 2014/15).

5.2.2. Treatments and design

Treatments consisted of four different groups of near isogenic lines (NILs) pairs each of them with lines contrasting for the introgression of *Eps*–early and –late alleles. Near isogenic lines

were developed at John Innes Centre (Norwich, UK), as described by Zikhali *et al*. (2014) and Farré *et al*. (2016). Three of these groups of NILs pairs were based on the cross Avalon x Cadenza (AxC) and the fourth group were NILs pairs based on the cross Spark x Rialto (SxR). One of the three groups of NILs pairs from AxC had the contrasting *Eps* alleles on chromosome 1DL, is composed of 6 lines with *Eps*–early and 6 lines with *Eps*–late (all in all 12 lines), whilst in the other two groups the contrasting *Eps* alleles were in chromosome 3AL (one of them with the background of Avalon- second group- and the other with the background of Cadenza- third group-). Second group consisted of 2 lines with *Eps*–early and 2 lines with *Eps*–late and the third group had 4 lines with *Eps*–early and 4 lines with *Eps*–late alleles. Finally, the fourth group is represented by NILs made with *Eps* effect on chromosome 1DL on the background of Rialto and consisted of 4 lines with *Eps*–early and 4 lines with *Eps*–late alleles (Table 5.2). Within each of the groups of NILs the different lines differed only in the DH lines used to develop the NIL pair, and we grew them separately just to be able to identify any unforeseen differences between lines with the same *Eps* allele. But, as expected all differences were only ascribed to the effect of the specific *Eps* gene. Therefore, we finally analysed the effect of each specific *Eps* within each group of NILs as the average in the variables analysed between all lines sharing the same *Eps* allele.

We did not get the seeds of the NILs pairs from AxC with the contrasting *Eps* alleles on chromosome 3AL in the first year. Therefore, the first growing season for these NILs coincided with the second growing season of the other NILs pairs. This did not represent any inconvenience for analysing the effect of particular *Eps* genes within each of the crosses.

Table 5.2. Groups of NILs pairs used in this experiment and their description (donors, recurrent parents, chromosome where QTL was identified, alleles (late = allele conferring lateness*;* early = allele conferring earliness) and number of replicates used in the experimental design.

Group of NILs pairs	Donors	Recurrent parent	Chromosome of QTL	Alelle	Growing season	Number of replicates
1	Avalon x Cadenza	Cadenza	1D	*Eps*- late	2012-13 2013-14	18
				Eps- early		18
2	Avalon x Cadenza	Avalon	3A	*Eps*- late	2013-14 2014-15	6
				Eps- early		6
3	Avalon x Cadenza	Cadenza		*Eps*- late		12
				Eps -early		12
4	Spark x Rialto	Rialto	1D	*Eps*- late	2012-13 2013-14	12
				Eps -early		12

Although groups 2 and 3 had different recurrent parents, all NILs with the same *Eps* alleles were analysed together and the effect of the *Eps* gene in 3AL determined with the average of eighteen replicates for each one of them. We disregarded the difference between backgrounds because the interaction between *Eps* alleles and recurrent parent was not significant (*P=0.998*).

Treatments were arranged in a completely randomised design with eighteen (for NILs from AxC) and twelve (NILs coming from SxR) replications, as each line had three replicates. Plots were 5 m long with six rows 0.20 m apart (in the 2012/13 and 2013/14 growing seasons) and 4.0 m long with six rows 0.20m apart (in 2014/15).

5.2.3. Measurements and analyses

Phenological stages were determined according the decimal code developed by Zadoks *et al*. (1974). At seedling emergence, three plants per experimental unit were selected and labelled in order to monitoring leaf and tiller appearance following the scale proposed by Haun (1973). All in all, the periodic determinations of leaf and tiller number were done in 54 plants of each NIL of the AxC cross and in 36 plants of each NIL of the SxR cross. From seedling emergence to anthesis, the number of leaves and tillers appeared were recorded frequently (from once to twice per week, depending on temperature). In total there were 20 determinations of leaf and tiller numbers in each of the labelled plants and with these values the dynamics of leaf appearance (and estimates of phyllochron; i.e. thermal time elapsed between the appearance of two successive leaves) and tillering were analysed. One representative plant per plot was randomly sampled and taken to the lab (from one to three times per week, depending on temperature), where they were dissected under a binocular microscope (Leica MZ 7.5, Leica Microsystems, Heerbrugg, Switzerland) to (i) determine the developmental stage of the apex according to Waddington *et al*. (1983), and (ii) count total number of leaf and spikelet primordia. All in all there were 31 samplings and with these records the dynamics of leaf and spikelet primordia initiation were analysed (and leaf- and spikelet-plastochron, thermal time elapsed between the initiation of two successive primordia in the apex, estimated).

Dynamics of leaf appearance and of primordia initiation were obtained by plotting the Haun stage and the number of (leaf and spikelet) primordia against thermal time, respectively (Fig. 5.2). In both cases, data distribution clearly evidenced bilinear trends, and therefore data were fitted through bilinear regression. For leaf appearance it was clear that early leaves appeared

at a higher rate (shorter phyllochron) than late leaves (Fig. 5.2A). For primordia initiation, it was evident that the initial (leaf) primordia were initiated much slower than the later (mostly spikelet) primordia (Fig. 5.2B). Phyllochrons (of early- and late-appearing leaves -Phy I and PhyII, respectively), and plastochrons (leaf- and spikelet-plastochron) were calculated as the reciprocal of the slopes. The timing of the break in rate of leaf appearance was fixed at the emergence of the seventh leaf (Slafer and Rawson, 1997) (Fig. 5.2A). For the primordia initiation dynamics the bilinear model was fitted with a fixed intercept (4 primordia, corresponding to the 4 leaves initiated during seed filling in the mother plant and therefore already developed in the embryo of the seeds sown) (Fig. 5.2B). Dynamics of tillering (including tillering and tiller mortality) were analysed using a tetaralineal model, where rate of tiller appearance was high firstly, null later, reaching a plateau (maximum number of tillers), and negative later representing the rate of tiller mortality until final number of tillers per plant (spikes per plant) was determined (Fig. 5.2C).

Timing of floral initiation was estimated *a posteriori* when FLN had been determined. Double ridge and terminal spikelet stages were determined under dissection based on Waddington *et al*. (1983) (Fig. 5.2B).

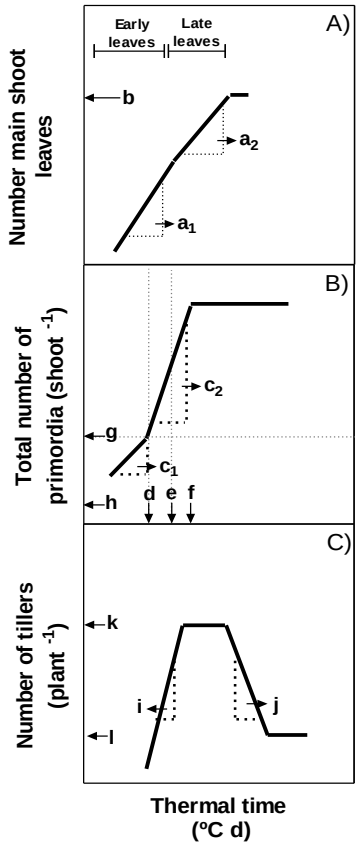


Fig. 5.2. Schematic diagram of the relationship between leaf number (A), leaf and spikelet primordia (B), or tiller number (C) and thermal time fitted to bilinear and tetralinear models, respectively to describe the dynamics of leaf appearance (a_1, phyllochron of early leaves; a_2,phyllochron of late leaves and b, final leaf number), dynamics of primordia initiation (c_1, leaf plastochron; c_2 spikelet plastochron; d, floral initiation; e, double ridge; f, terminal spikelet; g, final leaf number and h, number of primordia in the embryo at sowing) and dynamics of tillering (i, rate of tiller appearance; j, rate of tillering mortality; k, maximum number of tillers and l, number of fertile tillers).

A mixed model was fitted for all the traits within each group of NILs through JMP® Pro Version 12.0 (SAS Institute Inc. Cary, NC, USA). The model includes growing seasons, genotypes, and their interaction which were considered as fixed factors, and replicates nested with growing season as random. Differences between *Eps* alleles were tested using a post-hoc analysis of LSMeans contrast.

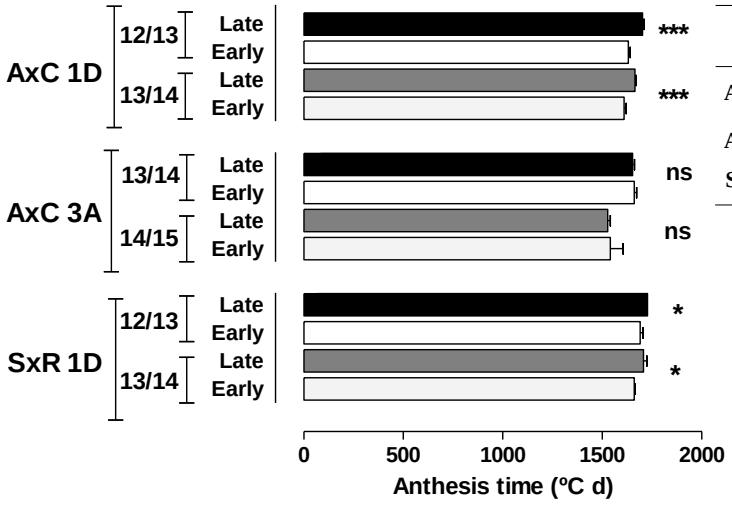
5.3. Results

5.3.1. Phenology

The duration of the whole cycle from seedling emergence to anthesis was significantly affected by the *Eps* alleles in chromosome 1D in both crosses, though the effect was larger in

AxC than in SxR (Fig. 5.3). Expectedly the magnitude of the effect was relatively small, still larger in AxC than in SxR (averaging across seasons *c*. 60ºC d in the AxC cross and *c*. 40ºC d in the SxR cross; Fig. 5.3). The difference on the magnitude of the effect for the "same" gene, which was consistent across both growing seasons, reflects either actual differences in the *Eps* gene -depending on the donor- and/or the effect of the interaction of this gene with the genetic background (Cadenza or Rialto). On the other hand and unexpectedly, the *Eps* alleles on chromosome 3A did not affect time to anthesis in any of the two growing seasons (Fig. 5.3); in fact, the NILs with the "late" allele tended to flower very slightly earlier than those with the "early" allele.

Although there was a difference in time to anthesis between growing seasons (albeit its magnitude was much smaller than that of the *Eps* gene in 1D), the interaction between both factors was absolutely negligible compared to the magnitude of the effects of the *Eps* genes and not significant in the three cases analysed (Fig. 5.3). This reinforced the consistency of both the effect of the *Eps* gene in 1D and the lack of effect of the *Eps* gene in 3A. Therefore, the results of the effects (or lack of effects) of these genes in the more detailed developmental traits considered from now on will be shown with the data averaged across both growing seasons.



	Growing season	*Eps* alleles	GS x *Eps*
AxC 1D	15,507 ***	68,886.6***	1,302.8 ns
AxC 3A	272,187***	1,892.5 ns	135.3 ns
SxR 1D	6,223.7*	16,177.6**	38.9 ns

Fig. 5.3. Duration (in thermal time) of the whole period from sowing to anthesis for late (black and dark grey bars) and early (white and light grey bars) alleles of different NILs within each of the two growing seasons in which each population of NILs were grown. NILs differed for the *Eps* alleles (early or late) in chromosomes 1D or 3A of the cross of Avalon x Cadenza or in chromosomes 1D of the cross of Spark x Rialto. The side table shows the mean squares of the growing season, *Eps* alleles and their interaction. segments on the bars stand for the SEMs and the asterisks indicate the level of significance of either the effects of the sources of variation (side Table) or the differences between

Eps-early and –late alleles within each group of NILs and growing season (**P<0.05*, ****P<0.001* and ns-not significant).

When analysing the *Eps* gene effects on the three sub-phases determining time to anthesis the results were not completely consistent. For the *Eps* genes in chromosome 3A the lack of effects on time to anthesis was simply reflecting the lack of effects in all sub-phases rather than opposite effects on them (Fig. 5.4, squares). The effects on time to anthesis of the *Eps* genes in chromosome 1D were evident in the late reproductive phase for both AxC and SxR NILs, but the effect was also clear in the vegetative phase for the *Eps1D* of SxR (Fig. 5.4 triangles) or in the early reproductive phase for the *Eps1D* of AxC (Fig. 5.4 circles). Recapping briefly, the *Eps3A* that had not affected total time to anthesis did not affect the duration of any of the component phases, whilst the *Eps1D* that significantly affected time to anthesis did so mainly through affecting the length of the late reproductive phase. Consequently, overall the *Eps* genes considered -and groups of NILs- the differences in the whole period from seedling emergence to anthesis were much better explained by those in the late reproductive phase (Fig. 5.4C) than in any of the other earlier phases (Fig. 5.4A,B).

In all cases, the stage of double ridges was reached much later than floral initiation (averaging across all genotypes, 256 ºC d later), evidencing that the first spikelets were initiated as single ridges, anatomically indistinguishable from leaf primordia under the microscope (Fig. S5.1). Only the *Eps1D* of AxC, that had affected the duration of the early reproductive phase did also affect the period from floral initiation to double ridge (Fig. S5.1 inset).

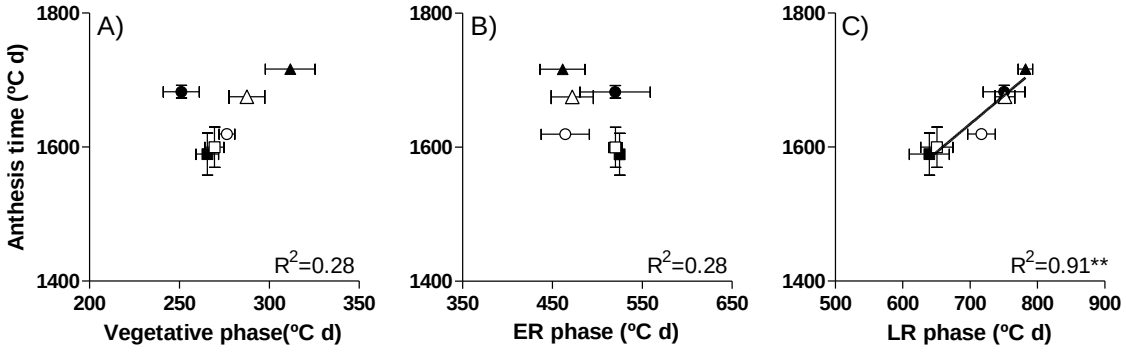


Fig. 5.4. Relationship between the duration of the period from seedling emergence to anthesis and each of its component developmental phases: vegetative (A), early reproductive (ER, B), late reproductive (LR, C) for NILs carrying either late (closed symbols) or early (open symbols) alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs, squares AxC 3A NILs, and triangles SxR 1D NILs. Data are averaged across two growing seasons. Segments on each symbol are the SEMs.

5.3.2. Final leaf number and phyllochron

The dynamics of leaf appearance was well described by bilinear models in all the six combinations of genotypes, 3 pairs of NILs with either *Eps*-early or –late alleles (Fig. 5.5; R^2>0.993, *P>0.001*). However, we did not find any clear effect of the *Eps* genes in any of the NILs pairs in any of the major parameters of the dynamics of leaf appearance (Fig. 5.5).

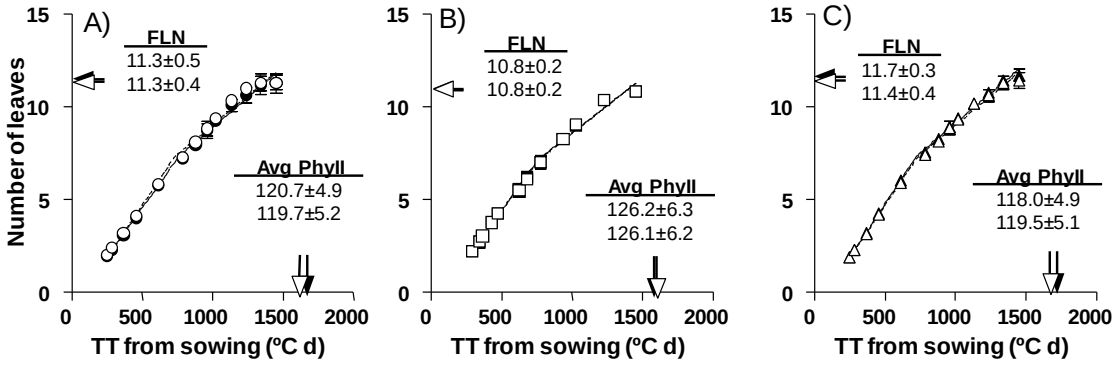


Fig. 5.5. Relationship between the cumulative number of appeared leaves in the main shoot (Haun stage) and thermal time after sowing for NILs carrying either late (closed symbols) or early (open symbols) alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs (panel A), squares AxC 3A NILs (panel B), and triangles SxR 1D NILs (panel C). Data are averaged across two growing seasons. Arrows (closed and open arrow-heads representing *Eps*-early and late alleles, respectively) indicate the timing of anthesis on the abscissa and the final leaf number on the ordinate. Inside each panel are the figures corresponding to final leaf number and averaged phyllochron for the *Eps*-late (top) and early (bottom) alleles.

There were no significant differences in final leaf number or in phyllochron between *Eps*–late and –early alleles in any of the three groups of NILs studied (Fig.5.5). However, there was a slight trend of reducing either phyllochron in AxC 1D (Fig. 5.5A) or FLN in SxR 1D (Fig. 5.5C) due to the action of *Eps*–early allele.

The analysis of the dynamics of leaf appearance is useful to understand the bases of the duration of the period from sowing to flag leaf emergence, but to understand the effects on time to anthesis we must also consider the duration between flag leaf appearance and anthesis. The variability on time to anthesis across the whole range of variation analysed in this study was actually explained by changes on both periods, sowing to flag leaf appearance and from then to anthesis (Fig. 5.6), though in both cases the relationships were driven by the differences between NILs pairs with some contribution from the differences within pairs due to the action of the *Eps* alleles. Focusing on the action of the *Eps* alleles, it is again clear that

Eps3A alleles did not affect either the time from sowing to flag leaf appearance nor that from flag leaf to anthesis, and that *Eps1D* alleles affected one or the other component of time to anthesis depending on the particular NIL pair considered. Thus, when analysing the effect of *Eps1D* from AxC it affected time to anthesis by changing the length of the both periods, from sowing to flag leaf appearance and from then to anthesis (Fig. 5.6, circles), whilst *Eps*-early alleles from SxR 1D advanced time to anthesis only through shortening the duration of the period to flag leaf appearance (Fig. 5.6A, triangles).

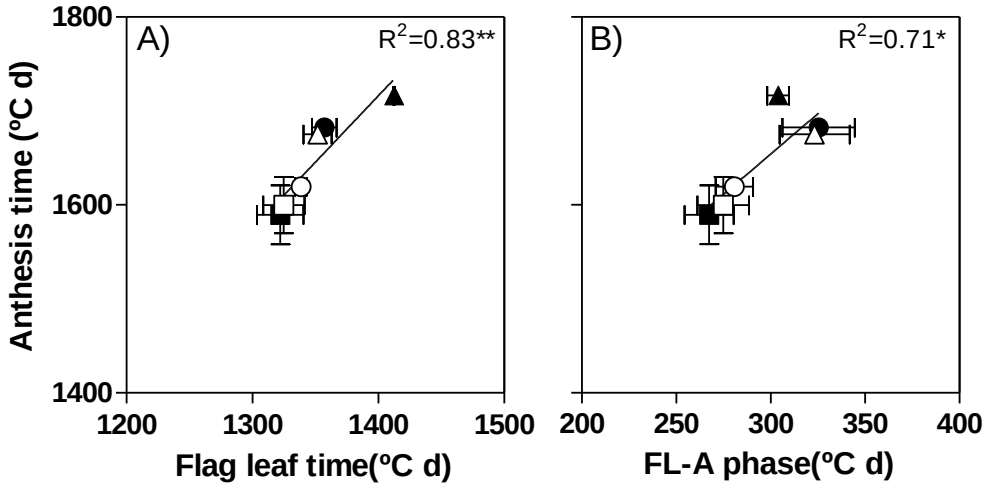


Fig. 5.6. Relationship between time from sowing to anthesis and that from sowing to the appearance of the flag leaf (A) or from then to anthesis (FL-A) (B) for NILs carrying either late (closed symbols) and early alleles (open symbols) of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs, squares AxC 3A NILs and triangles SxR 1D NILs. Data are averaged across two growing seasons. Segments on each symbol are the SEMs.

5.3.3. Spikelet primordia, leaf and spikelet plastochron and its coordination with phyllochrons

The dynamics of leaf and spikelet primordia initiation was expectedly well described by bilinear models, with an initial slower rate representing leaf primordia followed by a faster rate corresponding to spikelet primordia (Fig. 5.7; R^2>0.969, *P>0.001*). In line with the lack of effects on phenological development, the *Eps* gene in chromosome 3A did not affect the dynamics of primordia initiation at all (Fig. 5.7B). Consequently the plastochron of both leaves and spikelets and the final number of spikelets per spike initiated were virtually identical for *Eps3A* early and late alleles (Table 5.3).

On the other hand, the *Eps* gene in chromosome 1D did affect the dynamics but again the effects were not identical when comparing the NILs pairs of the AxC and SxR crosses. The

Eps-early allele of chromosome 1D from the AxC cross reduced the number of spikelet primordia significantly (decreased 0.8 spikelets per spike) (Table 5.4). Then, the reduction of early reproductive phase produced by this allele (Fig. 5.4B) resulted in a reduction in the number of spikelets differentiated, though of a lower magnitude as this allele also accelerated the rate of spikelet initiation (Fig.5.7A) reducing the spikelet plastochron. This effect on the spikelet initiation rate was also evident if considering the rate per emerged leaf instead of per degree day (Table 5.4), indicating that this *Eps* allele affected the coordination between primordia initiation and leaf appearance.

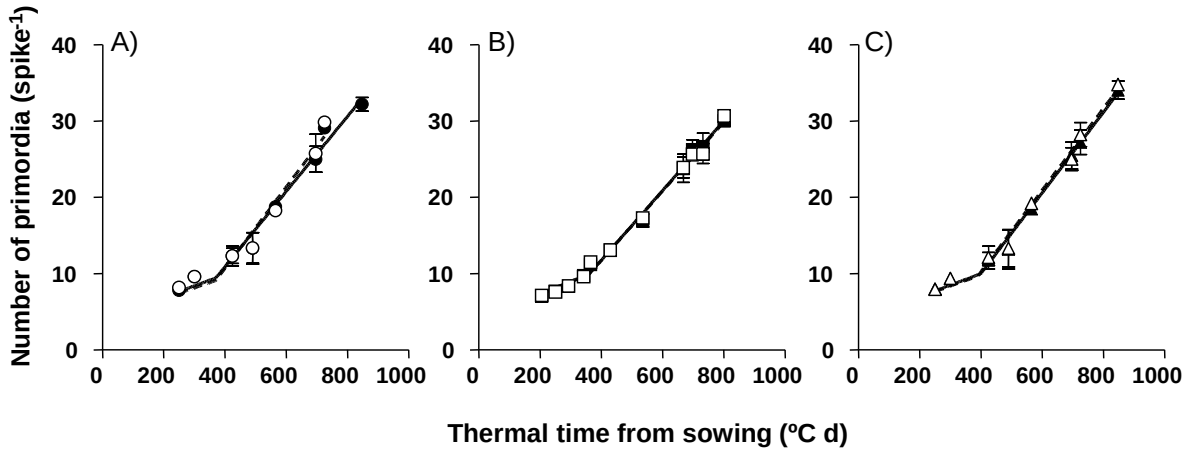


Fig. 5.7. Relationship between the cumulative number of primordia (leaves and spikelets) in the main shoot apex and thermal time after sowing for NILs carrying either late (closed symbols, plain lines) or early (open symbols, dashed lines) alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs (panel A), squares AxC 3A NILs (panel B), and triangles SxR 1D NILs (panel C). Data are averaged across two growing seasons.

On the other hand, the *Eps*-early allele of chromosome 1D from the SxR cross did not affect the number of spikelets per spike (Table 5.4), which is in line with the fact that this allele did not accelerate the developmental rate of the early reproductive phase (Fig. 5.4B). Although there was an apparent effect on the rate of spikelet initiation measured per degree days, it disappeared when considering the rate per emerged leaf (Table 5.4).

Table 5.3. Total number of spikelet primordia differentiated per spike and spikelet plastochron (both per degree day and per emerged leaf) for NILs carrying either late (closed symbols) or early alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) and in chromosome 1D of the cross of Spark x Rialto (SxR). Data are averaged across two growing seasons.

Donors	Chromosome of QTL	Allele	Spikelet primordia (spike^{-1})		Spikelet plastochron (^{o}C d spikelet^{-1})	Spikelet plastochron (emerged leaf $^{-1}$)
AxC	1D	Late	21.6±0.3	***	**20.3±1.4**	**7.1±0.5**
		Early	20.8±0.3		**18.8±1.4**	**8.0±0.6**
AxC	3A	Late	21.1±0.2	ns	21.9±1.3	4.2±0.3
		Early	21.0±0.1		22.1±1.4	4.1±0.5
SxR	1D	Late	23.1±0.4	ns	**18.9±0.8**	6.8±0.6
		Early	23.5±0.1		**21.1±0.9**	6.8±0.5

Values indicate mean±standard error of the mean (SEM). Bold values show that differences between alleles (late *vs* early) were significant based on their SEMs.

Beyond the effects of the *Eps* alleles it seemed consistently clear that the NILs from the SxR 1D had a "background effect" on the rate of spikelet initiation resulting in a shorter plastochron than that of the AxC 1D background (clearly seen when analysing in terms of spikelets per emerged leaf during the early reproductive phase) which resulted in a higher number of spikelets per spike (Table 5.4).

5.3.4. Tiller development and mortality

The dynamics of tillering, including tiller mortality, determining the maximum number of tillers and the final number of tillers (i.e. spikes) was either unaffected or only slightly and inconsequentially affected by the allelic form of the *Eps* genes considered (Fig. 5.8). Whenever the *Eps* alleles affected the maximum number of tillers produced (*Eps1D* and *Eps3A* from AxC), there was a trade-off with the tiller mortality rate causing the same number of fertile tillers (Fig. 5.8A and B). In the case of the *Eps1D* from SxR neither the maximum number of tillers nor the tiller mortality rates were affected at all (Fig. 5.8C).

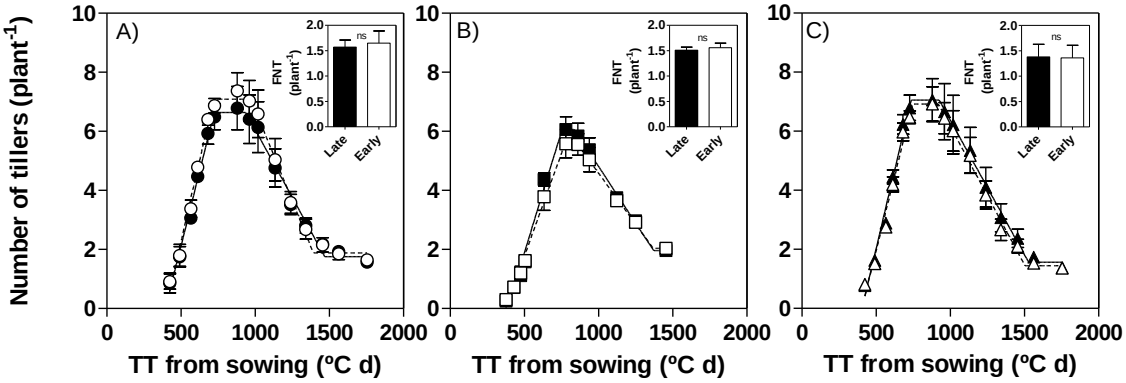


Fig. 5.8. Dynamics of the number of tillers per plant from sowing until the achievement of the final number of tillers (FNT) around anthesis (corresponding to the number of spikes, and included as an inset) for NILs carrying either late (closed symbols, plain lines) or early (open symbols, dashed lines) alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs (panel A), squares AxC 3A NILs (panel B), and triangles SxR 1D NILs (panel C). Data are averaged across two growing seasons.

If we used the number of phyllochron instead of thermal time as the independent variable, it can be possible to detect any effects of the *Eps* genes on the coordination between tiller and leaf appearance dynamics. Firstly, in all cases there was a clear coordination between tillering, until reaching the maximum number of tillers, and leaf appearance (Fig. S5.2), as in all cases the linear relationships between tiller and leaf numbers were highly significant (R^2>0.956, *P<0.001*). Secondly, the *Eps* genes did not modify the coordination between tillering and leaf appearance. Whenever there were apparent differences in either the slope (tillers appeared per phyllochron) or in the intercept on the abscissa (number of leaves appeared at the onset of tillering), they were not significant.

5.4. Discussion

Considering the overall effect of the Earliness *per se* alleles studied in the present study on the total time to anthesis there was a consistent effect of the *Eps*-late alleles in chromosome 1D reducing the rate of development that caused a delay in anthesis (though the magnitude of the effect was different depending of the cross generating the NILs) as well as a consistent lack of effect of the *Eps* gene in 3A. Griffiths *et al*. (2009) had found that both QTL *Eps* genes considered affected time to heading, which does agree with the results we found for the overall effects of *Eps*-1D but disagree with lack of effect of *Eps*-3A. Said that, it is also true that in Griffiths *et al*. (2009) the effects of *Eps*1D was not only stronger than those of *Eps*3A, but also the actual magnitude of the effect of the latter was very small. Although other studies

also reported QTLs for heading on chromosome 3A affecting time to heading (Shah *et al.*, 1999; Farré *et al.*, 2016), again the magnitude of the effects were virtually negligible. For instance, when analysing the effects in Farré *et al.* (2016), with the same lines used in our study (the work by Shah *et al*. (1999) compared chromosome 3A from Wichita and Cheyenne), the lines carrying the *Eps*-early allele in chromosome 3A headed in average only 25ºC d earlier than lines carrying the *Eps*-late allele (Fig. 5.9). Considering the *Eps*1D gene, our results also were of different magnitude than those reported in studies conducted in other locations with the same NILs (Fig. 5.9). The *Eps*-late alleles in chromosome 1D of AxC and SxR delayed time to flowering more or less than in Farré *et al.* (2016) or Zikhali *et al*. (2014), respectively (Fig. 5.9).

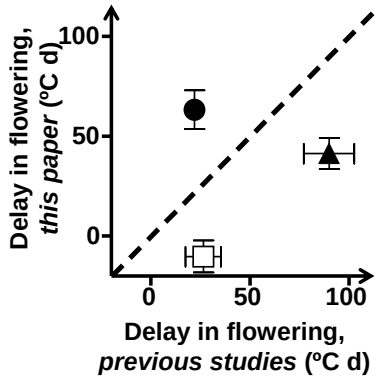


Fig. 5.9. Comparison of the magnitude of the effects of *Eps* genes in chromosome 1D of AxC (circle) or SxR (triangles) or 3A of AxC (square) grown under field conditions and under controlled conditions. The effects were estimated as the difference in thermal time from sowing to flowering (either anthesis or heading, depending on what was reported) between the NILs carrying late and early alleles. Results with the same lines grown in the field in the literature are those by Farré *et al.* (2016) for the AxC crosss and Zikhali *et al*. (2014) for the SxR cross. In the latter, data were reported in days after 1 May and were transformed to degree days using long term average temperatures in Norwich (where the study by Zikhali *et al*. (2014) was carried out). Dashed line is the 1:1 ratio.

All the quantitative discrepancies on the magnitude of effects of *Eps* genes reported in the current and in previous studies could be simply reflecting that the action of *Eps* genes is actually not "*per se*" but dependent upon the temperature condition in which they are expressed, as hypothesised initially long time ago (Slafer, 1996) and proven empirically thereafter (Bullrich *et al.*, 2002; Appendino and Slafer, 2003; Lewis *et al.*, 2008). For instance, the studies in which the lines carrying the *Eps*-early allele in chromosome 3A headed earlier were carried out on locations of N Europe which are noticeably cooler than the growing conditions in our experiments in Mediterranean Spain in which no effect was detected due to the action of *Eps*-3A.

Furthermore, although the *Eps*-1D gene had affected anthesis time, the magnitude of that

effect was stronger for NILs developed from the AxC cross than for those derived from the SxR cross. This highlights the influence of the genetic background of the genotype in which *Eps*-early or -late alleles may be introgressed to on the magnitude of the allelic effect. Some authors reported the possible *Eps* × background interaction (Farré *et al.*, 2016), which is not surprising as this sort of epistatic interaction is controlling time to flowering in cereals are well acknowledged (e.g. González *et al*., 2005*a*; Langer *et al*., 2014), although not always detected (Borras *et al*., 2010). Therefore, the actually expected effect of introgressing an *Eps* allele on a particular material to fine-tuning adaptation through slight changes in time to anthesis must be tested before hand on the specific germplasm (in addition to that it must be tested at the prevailing temperatures during the growing season).

Differences on anthesis time by *Eps* alleles were related with differences on late reproductive phase (period from terminal spikelet to anthesis). This findings offers to the breeders new tools to manipulate the duration of flowering time, affecting the late reproductive phase independently of preceding phases (vegetative and early reproductive phases). This has been hypothesised as a likely way in which developmental genes may be used to improve yield potential, beyond affecting adaptation (Slafer *et al*., 2001; Miralles and Slafer, 2007; Gonzalez-Navarro *et al*., 2016). This would be so because a longer late reproductive phase would increase the likelihood of floret primordia to end setting grains (e.g. González *et al*., 2003; 2005*b*; Serrago *et al*., 2008; Gonzalez-Navarro *et al*., 2016). This is relevant as most literature reporting genetic effects of *Eps* genes interpreted that the effect on time to flowering was due to an effect of these genes on the length of vegetative phase (e.g. Major 1980, Evans and Blundell, 1994).

When considering the effects of these alleles beyond phenology, we run out opportunities for discussing with other sources, as to the best of our knowledge this is the first time effects of *Eps* genes on detailed developmental processes are reported. As we did not find any effect of the *Eps*-3A genes in our study, we focused the interrelationship between traits affected by the *Eps*-1D genes. Not only the effect of this gene on time to anthesis was different for the two crosses analysed but also the cascade of developmental effects behind the overall response of time to anthesis were not identical.

When analysing the NILs of the AxC cross, the *Eps*-early allele

- did not consistently affect the developmental rates during the vegetative phase
- and therefore did not affect FLN

- although it did accelerate the rate of development of the early reproductive phases, reducing consequently the duration of the spikelet initiation
- but as it accelerated the rate of spikelet initiation as well (reducing spikelet plastochron)
- the number of spikelets per spike actually initiated was only marginally reduced
- despite the FLN was not altered, the rate of leaf appearance was marginally accelerated, reducing proportionally phyllochron
- and this resulted in a reduction of the duration of the late reproductive phase as leaves tended to appear more slowly
- though the effect was not limited to the appearance of the FL: the rate of development continued accelerating from then to anthesis reducing the final period of the late reproductive phase from the appearance of the FL to anthesis

On the other hand, when analysing the NILs of the SxR cross, the *Eps*-early allele

- accelerated rate of development slightly during the vegetative phase, consequently reducing its duration,
- but it simultaneously reduced slightly the rate of leaf primordia initiation, resulting in a slightly longer phyllochron
- both effects were small and together marginally reduced final leaf number
- which in turn determined that the number of leaves that must appear was slightly less and that reduced the time to the appearance of the FL (as phyllochron was not affected by this particular gene)
- that resulted in a slightly affected duration of the late reproductive phase, even though the duration of FL to anthesis phase was not affected.

Regarding the two 'yield components' considered in this developmental study (number of spikes and number of spikelets per spike), in no case there were effects on the number of spikes. This lack of effect of genes having relatively small effects is not surprising as the number of spikes would be a yield component with very large plasticity (Sadras and Slafer, 2012) functioning as a coarse-tuning components useful for accommodating rather large changes in growing conditions (Slafer *et al*., 2014). The other yield component considered showed either independence of the allelic state of the *Eps* genes (*Eps* genes in 3A and in 1D of SxR) or only a marginal sensitivity (*Eps*1D of AxC). In the latter case, *Eps early* alleles reduced the number of spikelets per spike. Thus, if this *Eps* gene were used fine-tuning flowering time it might potentially have an indirect effect on yield potential (Lewis *et al*., 2008). Said that, it is also relevant to emphasise that the effects on this yield component

would be more marginal than that on development, as the effect of the gene was seen on both the rate of development during the early reproductive phase (reducing the duration of the spikelet initiation phase) and the rate of spikelet initiation (reducing the spikelet-plastochron). In agreement with our results, Lewis *et al*. (2008) also found a trade-off between the duration of spike development phase and the number of spikelets per spike: the *Eps*-late alleles noticeably lengthened the spikelet initiation phase but also lengthened the spikelet plastochron and consequently the increase in the number of spikelets per spike was relatively small.

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5.6. Supplementary material

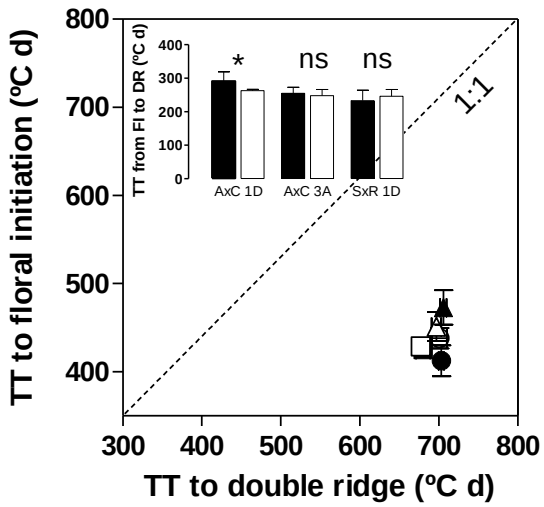

Fig. S5.1. Relationship between thermal time from sowing to either floral initiation or double ridge for late (closed symbols) or early (open symbols) alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs, squares AxC 3A NILs, and triangles SxR 1D NILs. Inset is the thermal time elapsed between the two stages for each of the genotypes. Data are averaged across two growing seasons. Segments on each symbol are the SEMs. Asterisks indicate the level of significance of the differences between *Eps*–early and –late alleles within each group of NILs (**P<0.05* and ns-not significant).

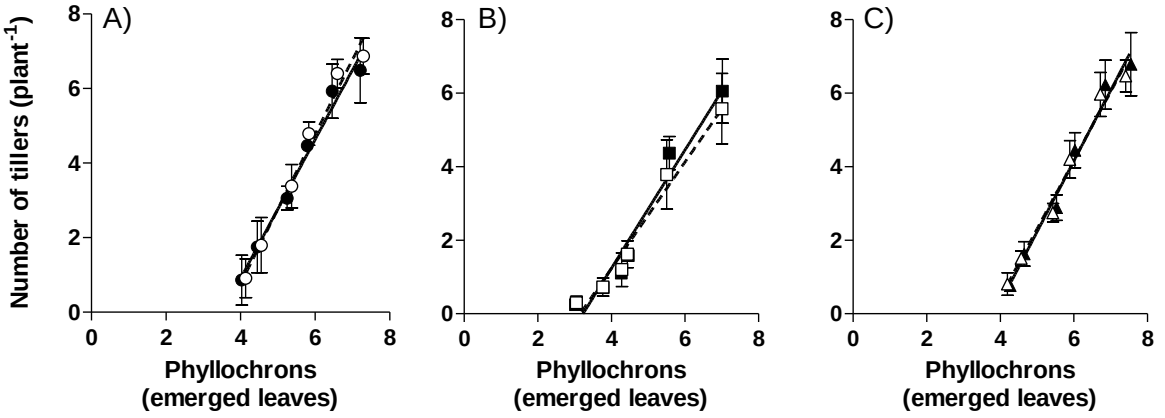

Fig. S5.2. Dynamics of tillering (from the onset of tillering to reaching the maximum number of tillers) based on phyllochrons (number of leaves appeared) for NILs carrying either late (closed symbols, plain lines) or early (open symbols, dashed lines) alleles of *Eps* genes in chromosome 1D or 3A of the cross of Avalon x Cadenza (AxC) or Spark x Rialto (SxR). Circles represent AxC 1D NILs (panel A), squares AxC 3A NILs (panel B), and triangles SxR 1D NILs (panel C). Lines were fitted by linear regression. Data are averaged across two growing seasons.

Chapter VI

Earliness per se x temperature interaction on wheat developmental traits

6. Chapter VI: *Earliness per se* x temperature interaction on wheat developmental traits

6.1. Introduction

Differences on time to flowering in wheat are critical for adaptation and improving yield potential (Worland, 1996; Slafer, 2003; Reynolds *et al*., 2012; Fjellheim *et al*., 2014). Genotypic variation of flowering time is mainly caused by differences in responses to photoperiod, vernalisation and temperature (Hay and Kirby, 1991; Slafer and Rawson, 1994). Genetic variation in sensitivity to photoperiod and vernalization (given by major genes: *Ppd* and *Vrn*, respectively) cause the largest effects on development being particularly useful for coarse-tuning adaptation (Griffiths *et al.*, 2009). However, there are still variations on flowering time once vernalization and photoperiods are totally satisfied; these differences have been termed as basic vegetative period, narrow sense earliness or intrinsic earliness and are produced by plants differing in their basic development rates or earliness *per se* (Slafer, 1996). These residual differences on time to flowering independent of photoperiod and vernalisation are regulated by earliness *per se* (*Eps*) genes (Snape *et al*., 2001). Main distinct characteristics of *Eps* –compared to *Ppd* and *Vrn*- genes are that there are many, distributed almost all over the genome (Snape *et al*., 2001; Kamran *et al*., 2014; Lopes *et al*., 2015), polygenic in nature (Rousset *et al*., 2011) and that their effects are rather small (Griffiths *et al*., 2009; Zikhali *et al*., 2014), but critical for fine-tuning developmental patterns (Snape *et al*., 2001; Lewis *et al*., 2008; Zikhali and Griffiths, 2015). Thus, there is a large degree of variation in *Eps* genes in germplasm of different regions (Worland *et al*., 1994; Appendino *et al*., 2003). Precisely because of the large number of *Eps* genes and their relatively small effect on development, the understanding of these genes and their effects are much poorer than that of *Ppd* and *Vrn* genes (Snape *et al*., 2001). Due to its little understanding there are several assumptions normally done on their action. One of them is reflected in the terms used to name the trait. The "intrinsic" in the most common description of the trait (intrinsic earliness) and the "*per se*" in the most common denomination of the genes controlling the trait (earliness *per se*) reflect the belief that the expression of the trait and the effect of the genes are "constitutive" and therefore independent of the environment (Worland *et al*., 1994; Snape *et al*., 2001). However, it seems that the belief was based on assumptions rather than on rigorous testing that independence.

Slafer (1996) put forward the hypothesis that these "*per se*" genes would likely be temperature sensitivity genes. Temperature has an universal effect on wheat development in

that all phases and all cultivars develop faster under high than under low temperatures (Slafer *et al*., 2015), a universal effect that can be seen well beyond wheat in the developmental rates of other living organisms not controlling their own temperature (e.g. Gillooly *et al*., 2002; O'Connor *et al*., 2007). But the fact that all cultivars and all phases are sensitive to temperature does not necessarily imply that the sensitivity is invariable. When the rates of development (Slafer and Rawson, 1995*a*) and the rates of leaf appearance (Slafer and Rawson, 1995*b*) were compared after vernalising the seedlings and under long days for contrasting cultivars grown through a range of thermal regimes it was clear that all processes were sensitive but that there was differences in sensitivity amongst cultivars. As under the conditions of the experiments all genotypic differences should have been due to their *Eps* genes, it was inferred that these genes would actually be temperature sensitivity genes (Slafer, 1996). Later on, working with an *Eps* gene discovered in *Triticcum monococcum* that had an unusually large effect on time to heading (*c.* 60 d difference between lines with the *Eps-Am1*-late and -early alleles; Lewis *et al*., 2008), it was confirmed the *Eps* x temperature interactions (Bullrich *et al*., 2002; Appendino and Slafer, 2003; Lewis *et al*., 2008) that had been suggested in the earlier studies with cultivar comparisons.

Results from that study with an unusually strong *Eps* gene in a diploid species can be hardly extrapolated directly to normal small-effect *Eps* gene in hexaploid wheat. Regretfully, there are no studies reporting *Eps* × temperature interaction in different developmental traits on hexaploid wheat. This assertion is true for overall development determining time to heading, not to mention for more detailed developmental traits. Although there are evidences that ambient temperature affects different developmental traits (e.g. Slafer and Rawson, 1995, Karsai *et al*., 2013), the specific interaction between *Eps* and temperature beyond time to heading has not been reported. A more quantitative approach about how the ambient temperature modulate earliness *per se* effects on pre-flowering phases is essential to fine-tuning the adaptation and to optimise yield potential, because in these pheno-phases is being determined source and sinks contributing to yield (both number of leaves and number of spikelets contribute to yield). Number of spikelets per spike seemed not to be modulated by interaction between temperature and *Eps* alleles when considering the unusually large Eps gene of *T monococum* mentioned above (Lewis *et al*., 2008).

Studies carried out in the UK with NILs carrying either the Spark allele (*Eps*-early) or the Rialto allele (*Eps*-late) for the *Eps* quantitative trait locus (QTL) located on wheat 1DL showed a 3–5 days of difference in time to flowering. This agrees qualitatively with our results (Chapter V), in which NILs with the *Eps*-early allele reached flowering 41.4 ºC d

earlier than those with the *Eps-late* allele. However, quantitatively there was little agreement, using the same NILs from SxR the magnitude of the *Eps* effects were clearly stronger in the experiments conducted in the UK than in Spain. Being the UK growing environments much colder than those in Spain, this quantitative disagreement may be considered an indirect evidence of a possible effect of temperature on the action of *Eps* alleles.

Then, the general objective of this work was to detect and, if that were the case, to quantify the effects of *Eps*1D alleles derived from the SxR cross under a wide range of growing temperatures on phenology, dynamics of leaf appearance and dynamics of leaf and spikelet initiation.

6.2. Materials and methods

6.2.1. General conditions

The experiments were carried out in the facilities of both the University of Lleida (UdL) and John Innes Centre (JIC) using growth chambers (GER-1400 ESP, Radiber, S.A., respectively). Before starting the experiments in the growth chambers, all seedlings were vernalised and then during the experiment chambers were at long photoperiod. For vernalising the seedlings, pots (200 or 400 cm^3, at UdL and JIC, respectively) were filled with a mixture of 30% peat and 70% soil (UdL) and cereal mix compost composed of 40% peat and 40% soil and 20% grit (JIC) and seeds were sown at a rate of a single seed per pot. A 40-50% of extra pots were sown to choose at the beginning of the experiments the most uniform seedlings in order to minimise the experimental error due to plant-to-plant variations. After sowing and irrigation, pots were left at room temperature (*c*. 20 °C) during 1 day for the germination process to be triggered, the metabolic activity fully recovered (so that the vernalisation stimulus could be fully perceived). After that day, pots were located in a cool room at 4ºC in dark during 49 days. After 49 days, all the selected pots were transferred to the corresponding growth chamber, and we considered this timing as the onset of the experiment. Chambers were always set under long day conditions (18 h). Pots inside of chambers were rotated approximately once a week to minimise likely differences in microenvironments at different positions within chambers.

6.2.2.Treatments

The treatments consisted of factorial combinations of two sets of near isogenic lines (NILs) carrying different *Eps* alleles and 7 temperature regimes.

- NILs were developed at John Innes Centre (Norwich, UK) from two double haploid lines derived from the cross Spark × Rialto (SR9 and SR23, both carrying the *Eps*-early allele of Spark in chromosome 1DL) as described in Zikhali *et al*. (2014). Briefly, initially each of these lines was back-crossed twice with the recurrent parent Rialto (which carries the *Eps*-late allele in 1DL) producing, within each of the two DHs families, BC_2 progenies (equivalent to BC_3 in terms of recurrent parent background composition). These BC_2 lines were self-pollinated producing BC_2F_3 families, and within these families lines that proved homozygous for *Eps* alleles on chromosome 1DL (carrying the *Eps*-early or -late) were selected using SSR markers. Each NIL pair was then composed of a NIL carrying *Eps*-late allele and another carrying *Eps*-early allele, within the families derived from the SR9 and SR23 lines.
- Temperature treatments (always maintained constant during the whole day and throughout the experiment) of 24, 21, 15, 9 and 6°C were imposed at the UdL whilst the temperatures regimes of 18 and 12°C were imposed at JIC.

Fifty-five (UdL) or 63 (JIC) pots per genotype were arranged inside each chamber (temperature regime) in a completely randomised design with three replicates. Each replicate was formed by 1/3 of the pots corresponding to each NIL, that is, 18 (UdL) and 21(JIC) pots per replicate and per genotype, resulting a total of 220 (UdL) and 252 (JIC) pots.

6.2.3.Measurements and analyses

Three plants per replicate of each genotype (all in all 9 plants per genotype) were labelled at the beginning of the experiments and on them we determined the phenological stages of seedling emergence (stage DC10), flag leaf emergence (DC39), heading (DC59) and anthesis (DC65) following the Decimal Code developed by Zadoks *et al*. (1974) and phyllochron. For the latter, we determined leaf appearance dynamics, from the initiation of the experiment (when vernalised seedlings were moved to the chambers), onwards the number of leaves appeared on the main shoot was recorded frequently, following the scale of Haun (1973). The actual frequency depended on the temperature from one to four times per week. Leaf appearance dynamics presented bi-linear trends with the break-point at around the appearance of the 7^{th} leaf in the most of temperature treatments (except to 6 and 9°C regimens). For this reason, two different phyllochrons were calculated for each combination of genotype and temperature, as the reciprocal of the first slope (phyllochron of early leaves) and of the second slope (phyllochron of late leaves) of the bilinear regressions. However to account for

overall effects of treatments, we also calculated a weighted average phyllochron.

From the other unlabelled pots, we took destructive samples along the experiment. One plant per replicate of each genotype was randomly harvested and dissected to determine the apex stage of development following the scale of Waddington *et al*. (1983) as well as to count the number of primordia in the apex allowing the determination of primordia initiation dynamics. For this purpose, the cumulated number of primordia was related with time since the onset of the experiment and the data fitted with bilinear regressions. From the bilinear regression, plastochrons (leaf- and spikelet-plastochron) were calculated as the reciprocal of the first and second slope, respectively. After determining the stage of terminal spikelet we divided the duration of the period from the onset of the experiment to heading into two phases (i) from the onset of the experiments to terminal spikelet, including the vegetative and early reproductive phases, and (ii) from then to heading, comprising the late reproductive phase.

Data of variables were subjected to analysis of variance and the relationships describing dynamics or relationships between traits were analysed through regression analysis (depending on the particular cases we used linear or bi-linear regressions, in order to maintain a random distribution of residuals). All analyses were performed using statistical software JMP® Pro Version 12.0 (SAS Institute Inc. Cary, NC, USA). Differences among *Eps* alleles in each temperature treatment were tested using ANOVA and a *post-hoc* analysis of LSMeans Contrast.

6.3. Results

6.3.1.Abnormalities in development

When plants were grown at both extremes of the temperature range included in the study (6, 21 or 24°C) the patterns of development exhibited different types of abnormalities, none of which were visible in any of the plants grown at all other thermal conditions (9, 12, 15, and 18°C).

Plants grown at 6°C never reached anthesis, despite that they developed apparently normal, though slowly, until heading. Failure in reaching anthesis would hardly be due to lack of enough experimental time. Firstly, the experiment was maintained for 89 d after heading of the NILs carrying the *Eps*-late alleles (101 d after heading of the *Eps*-early NILs) and even in such long period for an intrinsically short phase none of the plants reached anthesis.

Secondly, florets inside the spikelets at this thermal regime seemed to have developed an abnormal morphology: e.g. the anthers lost their colour and the florets seemed to be aborted before pollination. In addition, apical spikelets started to lose colour before flowering. Consequently, although 100% of the plants reached heading normally, none reached anthesis when grown at the lowest temperature of the study (Fig. 6.1).

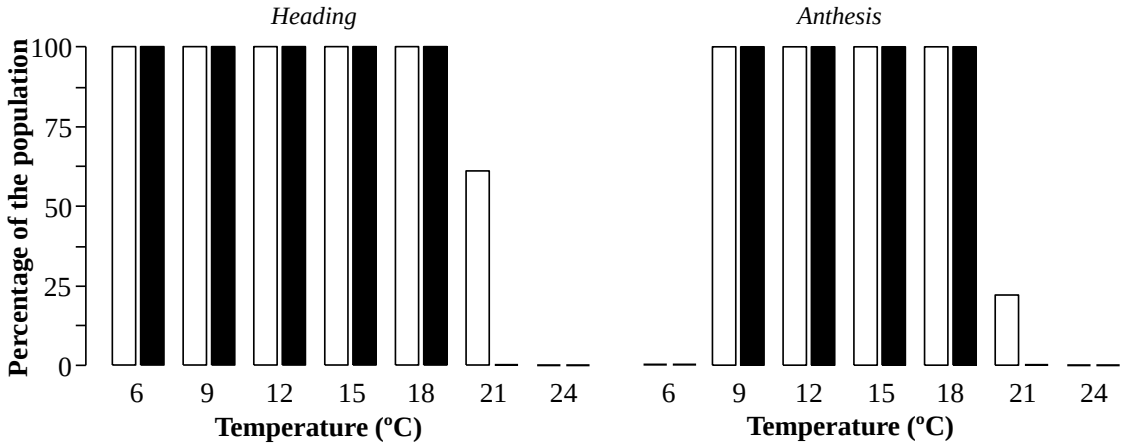

Fig. 6.1. Percentage of the population of plants with *Eps*-early (open bars) or *Eps*-late (closed bars) that reached heading (left) or anthesis (right) grown under constant temperatures of 6, 9, 12, 15, 18, 21 and 24 °C.

When plants were grown at 21°C none of the plants with *Eps*-late alleles and c. 60% of those with *Eps*-early alleles reached heading, and less than half of them did continue developing normally to reach anthesis (Fig. 6.1); but all plants reached the terminal spikelet stage; indicating that the impairment of development occurred during the late reproductive phase. At 24°C all plants, regardless of their *Eps* allele developed normally until the appearance of the first 5-6 leaves, but then few following leaves appeared extremely slowly, the earlier leaves started to senesce prematurely and later leaves became clearly undersized, and finally none of the plants reached even the double ridge stage. In the rest of the results section, when analysing durations or rates of developmental processes we considered only the plants that reached the particular developmental trait. Due to the relatively large failure in reaching anthesis from plants that did reach heading normally, we used heading rather than anthesis as the most integrative developmental trait in the rest of the Results section. However, disregarding the growth at 6ºC (in which plants did not reach anthesis), heading time reflected perfectly well time to anthesis (Fig. S6.1). The very high coefficient of determination is due to the clear effect of temperature, reflecting that considering either of the two traits would be virtually the same when accounting for temperature effects. Also within thermal regimes lines carrying the *Eps*-late alleles showed in average longer times to both

stages than those with *Eps*-early alleles (closed symbols on the right and above the open symbols; Fig. S6.1). And the non-crossover *Eps* x temperature interaction (see below) was also clear in that the distance between open and closed symbols tended to be larger at 9ºC than at mild and warm temperatures. Therefore, general conclusions regarding the effects of temperature, *Eps* alleles and their interaction would be the same considering either of the two integrative traits.

6.3.2.Phenology

The plants having the same *Eps* alleles from the two pairs of NILs, derived from either SR9 or SR23, had exactly the same behaviour. Considering time to heading as the overarching trait embracing developmental processes in this study, it can be easily seen that the *Eps*-early NILs of each of the two families (SR9 and SR23) reached heading almost simultaneously at each of the temperature conditions in which this stage was reached, and the same was true for the *Eps*-late NILs of both pairs (Fig. 1). Naturally, this reflected the fact that the effect of the NIL pair (whether lines were derived from SR9 or SR23) was not insignificant, as well as its interaction with temperature (Fig. 6.2, inset). In fact, the linear regression of the relationship in Fig.6.2 was not only very highly significant but also had an intercept not different from the origin and a slope not different from 1 (Fig. 6.2). Therefore, in the rest of the paper the results of the two *Eps*-early and the two *Eps*-late NILs are averaged.

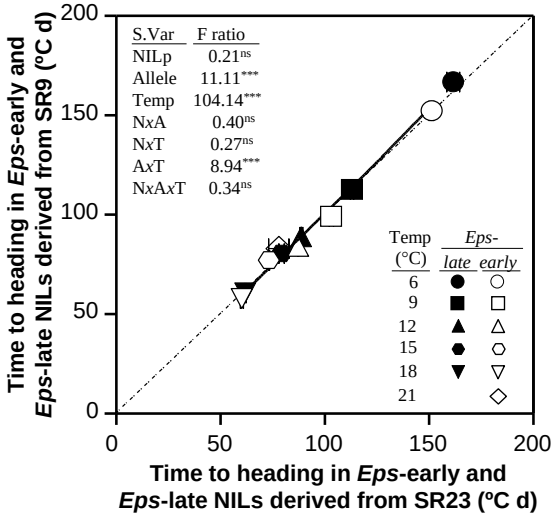

Fig. 6.2. Time from the initiation of the experiment to heading for the NILs carrying the same *Eps* alleles in each of the two NIL pairs (NILs derived from either SR9 or SR23, see Materials and Methods) under the temperature regimes in which heading was reached. Dashed line is the 1:1 relationship. Solid line fitted by linear regression (y = 1.03±0.03 x – 2.23±3.01; R^2=0.993, *P<0.001*).

Inset are the F-ratio values of the main factors and their interactions (*** and ns indicates that the F-ratio or the R^2 were significant, *P<0.001*, and not significant, respectively). Segments on each symbol stand for the SEM (if not seen is because the magnitude was smaller than the size of the symbol).

In general, it was clear that temperature accelerated development and that the *Eps*-early alleles accelerated development at all temperatures. This can be seen by the fact that the F-ratios of the main factors were much larger (temperature) and larger (*Eps* alleles) than that of the interaction (Fig. 6.2, inset). This is illustrated with pictures taken under microscope to dissected apexes at regular intervals after the onset of the experiment for NILs carrying the *Eps*-early or late alleles when grown under 12 and 18ºC (Fig. 6.3).

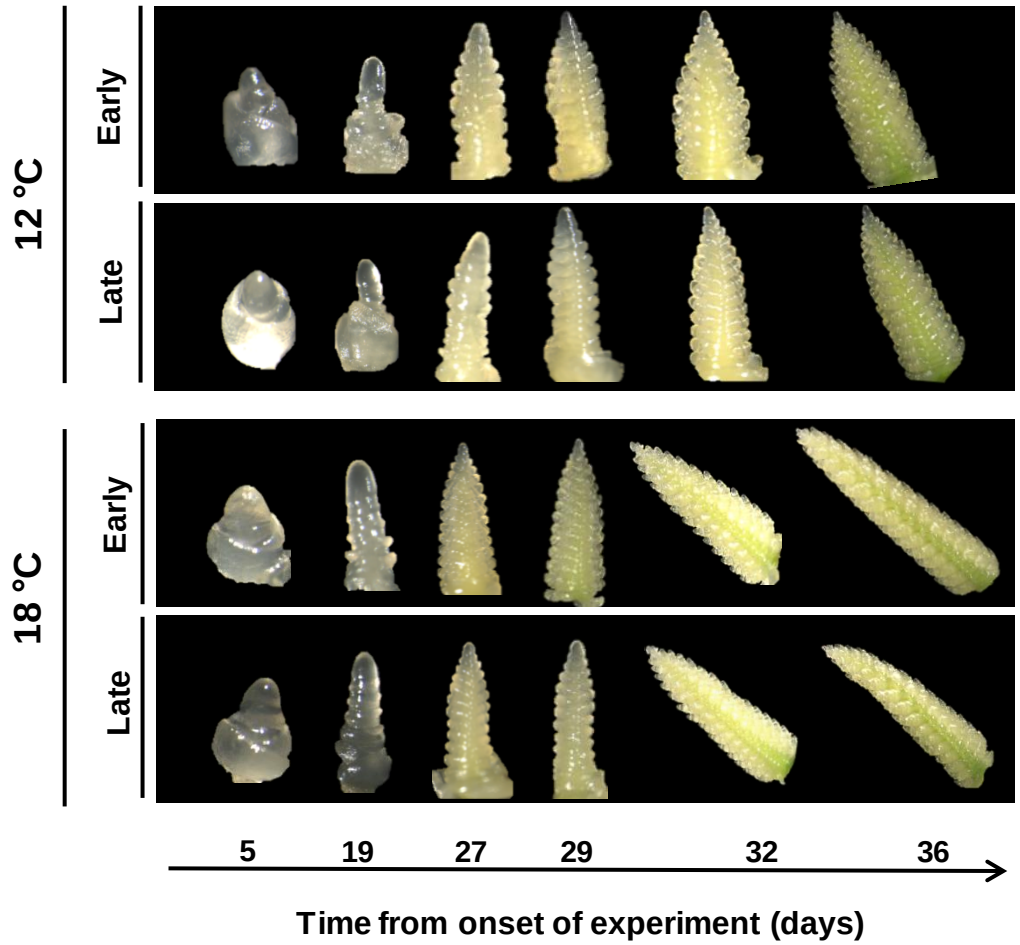

Fig. 6.3. Illustration of apex development from 5 to 36 d after the onset of the experiment for lines carrying *Eps*-early and -late alleles grown at constant temperature of 12 (top panel) and 18°C (bottom panel).

As expected, temperature increases accelerated developmental processes and consequently, in general, the length of the period from onset of the experiment to heading was shorter under warmer temperatures in which all plants developed normally until heading (Fig. 6.4). It was clear that when comparing the time to heading under 21 and 18ºC, the opposite was true:

plants headed earlier at 18 than at 21ºC (Fig. 6.4). It was also clear that lines carrying the *Eps*-late allele headed always later than those with the *Eps*-early allele (Fig. 6.4). However, the magnitude of the difference was not the same across temperatures: at low temperatures the effect of the *Eps* gene was clearly more noticeable than at mild and warm temperatures (Fig. 6.4, inset). This revealed a clear *Eps* x temperature interaction of quantitative nature: i.e. there was a difference in the magnitude of the effect, but the interaction was not crossover in nature as it lines with the *Eps*-late allele were never earlier than those carrying the *Eps*-late allele (Fig. 6.4), and as it was expected from the fact that the F-ratio of the *Eps* x temperature interaction was smaller than that of the direct effect of the allelic constitution and much smaller than that of temperature (Fig. 6.2, inset).

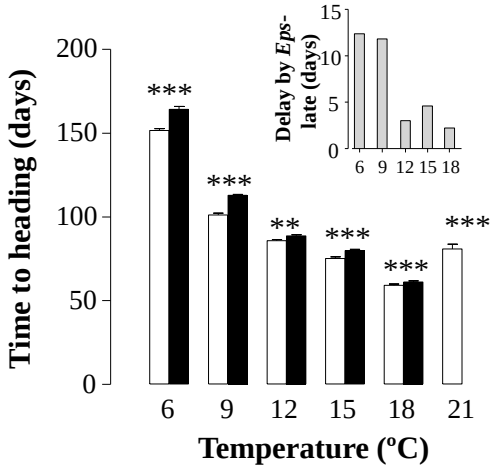

Fig. 6.4. Days from the onset of the experiment to heading for lines carrying *Eps*- early (open bars) or *Eps*-late (closed bars) alleles grown under constant temperatures of 6, 9, 12, 15, 18, and 21ºC (treatment at 24°C was not included as none of the plants at that condition reached heading). Inset is a detail of the difference between lines with the *Eps*-late or *Eps*-early alleles. Asterisks indicate the statistical significance of the differences between lines with *late* and *early* alleles at each temperature from the LSmeans contrast (***P<0.01*, ****P<0.001*).

Across all treatments, time to heading was well explained by the two component phases considered in the study: from the onset of the experiment to terminal spikelet (Fig. 6.5, left panel), and from then to heading (Fig. 6.5, right panel). Naturally the very high coefficients of determination are due to the universal effect of temperature: as all phases are sensitive, the R^2 of the relationship between duration of phases must be very high. However, the sensitivity of both phases was not identical: the time elapsed between the onset of the experiment and terminal spikelet ranged from 33 to c. 74 d (an increase of 220% in duration of the phase from 18 to 6ºC), whilst duration of the period from terminal spikelet to heading ranged from c. 26 to c. 91 d (an increase of 350% in duration of the late reproductive phase from 18 to

6ºC). In addition, the delay produced by the *Eps*-late allele (difference between plants carrying *Eps*-late and *Eps*-early alleles) in time to heading was better explained by the delay in the late reproductive phase (Fig. 6.4, inset right panel) than by that of the period from the onset of the experiment to terminal spikelet (Fig. 6.5, inset left panel). Thus, the effect of the main factors (temperature and *Eps* gene) as well as that of the *Eps* x temperature interaction shown in time to heading was more clearly reflected in the late reproductive phase than in the period from the onset of the experiment to terminal spikelet.

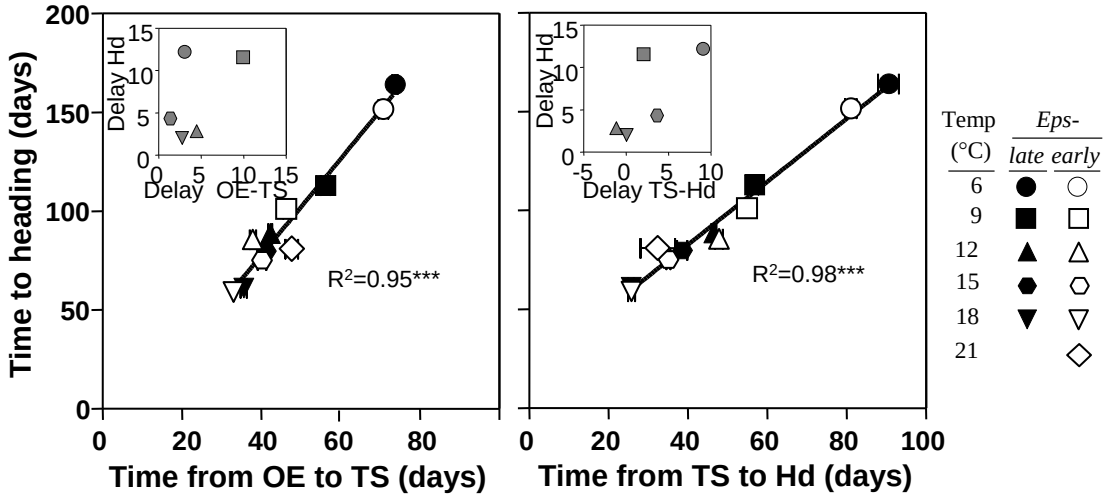

Fig. 6.5. Relationship between time to heading and either the period from the onset of the experiment (OE) to terminal spikelet (TS) (left panel) or the late reproductive phase from TS to heading (Hd) (right panel) for lines carrying *Eps*-early (open bars) or *Eps*-late (closed bars) alleles grown under constant temperatures of 6, 9, 12, 15, 18 and 21°C. Lines fitted by linear regression (**P<0.001*). Inset each panel is a detail of the delay produced by the *Eps*-late allele (difference between lines with the *Eps*-late or *Eps*-early alleles) in time to heading respect to the delay in the component phases considered in the study.

6.3.3.Leaf appearance and time from flag leaf to heading

Leaf appearance dynamics was strongly affected by growing temperature. In general, the leaves appeared very slowly at 6ºC (at *c*. 0.05 leaves day^{-1}) and the rate of appearance increased (shifting the data-points counter-clockwise) as the conditions became warmer until reaching a maximum rate at 18ºC (at *c*. 0.20 leaves day^{-1}) (Fig. 6.6A), but then leaves appeared at slower rates when grown at even warmer temperatures (e.g. at *c*. 0.15 and 0.12 leaves day^{-1} when grown at 21 and 24ºC, respectively). Oppositely to the effect of temperature, we did not find any consistent effect of *Eps* alleles on the rates of leaf appearance, and very minor effects were unpredictable (Fig. 6.6A).

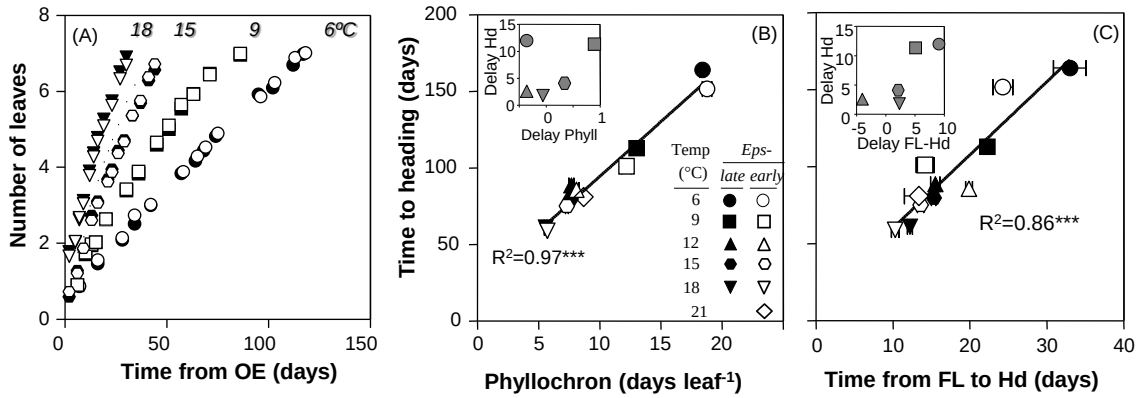

Fig. 6.6. Dynamics of leaf appearance (to illustrate more clearly restricted to the first 7 leaves of plants grown under selected temperatures) (A), and relationships between time to heading and either average phyllochron (B) or time from the appearance of the flag leaf (FL) to heading (Hd) (C) for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 6, 9, 12, 15, 18 and 21°C. Lines in panels B and C were fitted by linear regression. Inset each of the two last panels is a detail of the delay produced by the *Eps*-late allele (difference between lines with the *Eps*-late or *Eps*-early alleles) in time to heading respect to the delay in phyllochron (B) and in the duration of FL to Hd (C).

Time to heading was strongly related to both phyllochron (Fig. 6.6B) and time from the appearance of the flag leaf to heading (Fig. 6.6C). Once again the major driver for these strong relationships was temperature through its universal effect of developmental rates affecting simultaneously all traits considered in the same direction. The advancement of heading due to the action of *Eps*-early, instead of *Eps*-late, alleles could be hardly explained by its effect on phyllochron: the differences in phyllochron between NILs carrying *Eps*-late and *Eps*-early alleles were rather small and inconsistent (data-points of NILs with *Eps*-late alleles were inconsistently on the right or the left of those corresponding to NILs with *Eps*-early alleles under the same temperature; Fig. 6.6B), as well as unrelated to the differences between the NILs in time to heading (Fig. 6.6B, inset). On the other hand, data-points corresponding to NILs with *Eps*-late alleles were in general above and to the right of those corresponding to NILs with *Eps*-early alleles (Fig. 6.6C), although there was an exception at 12ºC. Thus, there was a positive relationship between the effect of the *Eps* alleles on time to heading and on the period from the appearance of the flag leaf and heading (Fig. 6.6C, inset), suggesting in general that the last part of the late reproductive phase would have been responsible for the effect of the gene.

6.3.4.Spikelet initiation and spikelets per spike

Temperature treatments also markedly affected the dynamics of spikelet initiation. The rate of

spikelet primordia initiation resulted slow at 6ºC (at *c*. 0.30 primordia day^{-1}) it increased with increasing temperatures until reaching a maximum rate at 18ºC (at *c*. 1.1 primordia day^{-1}) (Fig. 6.7A), and then spikelet primordia initiated at a slower rate at 21ºC (*c*. 0.41 primordia day^{-1}), while at 24ºC none of the plants reached floral initiation. Again, the effect of *Eps* alleles on the rates of spikelet initiation were not clear: in most cases differences between *Eps*-late and *Eps*-early NILs were negligible (see for instance the dynamics at 15 and 18ºC; Fig. 6.7A) or inconsistent (*Eps*-late lines had faster or slower rates of spikelet initiation than their *Eps*-early counterparts at 6 and 9ºC, respectively; Fig. 6.7A).

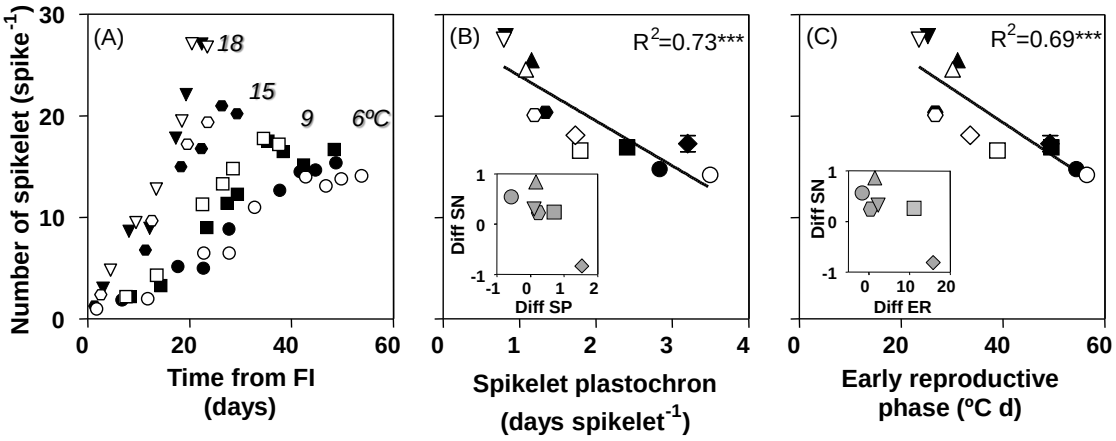

Fig.6.7. Dynamics of spikelet primordia initiation from floral initiation (FI) to terminal spikelet (to illustrate more clearly restricted to selected temperatures) (A), and relationships between the final number of spikelets per spike initiated and either spikelet-plastochron (B) or duration of the early reproductive phase from FI to terminal spikelet (C) for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 6 (circles), 9 (squares), 12 (triangles), 15 (hexagon), 18 (inverted triangles) and 21°C (diamond). Lines in panels B and C were fitted by linear regression (*P<0.001*). Inset each of the two last panels is a detail of the relationship between the differences (*Eps*-late minus *Eps*-early lines) in number of spikelets (SN) per spike and in spikelet plastochron (SP) (B) or in the duration of the early reproductive phase (ER) (C).

The final number of spikelets per spike was negatively related to both the spikelet plastochron (Fig. 6.7B) and the duration of the early reproductive phase, from floral initiation to terminal spikelet (Fig. 6.7C). Naturally the strong relationships were produced by temperature effects, as the differences between *Eps*-early and *Eps*-late NILs in spikelets per spike were relatively minor (Fig. 6.7). Within these minor differences it seemed that differences in spikelet plastochron (r=-0.82, *P<0.05*, Fig. 6.7B, inset) and in duration of the early reproductive phase (r=-0.84, *P<0.05*, Fig. 6.7C, inset) were similarly relevant.

These relationships reflect that the overall effects on spikelets per spike was small because of the strong compensation between the effects of both temperature and *Eps* aleles on both rates: the rate of development (producing increases in duration of the early developmental rate having *Eps*-late alleles or being exposed to low temperatures) and the effect on the rate of

spikelet initiation (producing increases in spikelet plastochron the same factors). In fact the relationship between spikelet plastochron and duration of the early reproductive phase was strong and positive (Fig. S6.2). The very high strength of the relationship is due to the fact that it represents compensations produced by both factors. Temperature universal effects were expected but it is relevant that the action of the *Eps* gene also generated a similar sort of compensation (R^2=0.88, *P<0.01*, Fig. S2, inset).

6.3.5. Developmental responses to temperature

The significant *Eps* gene x temperature interaction in duration of phases, phyllochron or plastochron implies that the responsiveness to temperature of the rates of development determining the differences in durations must be different for NILs carrying *Eps*-early alleles and late alleles.

We found that the rate of development towards heading was very well fitted to a bi-linear regression (R^2=0.993 and 0.807 for *Eps*-early alleles and late alleles, respectively). The relationship had a first positive slope (indicating that plants develop faster at higher temperatures between the base and the optimum thresholds) followed by a second negative one (indicating decline in developmental rates with increasing temperatures between the optimum and maximum thresholds) (Fig. 6.8). The parameters of the relationship revealed slight changes in base, optimum and maximum temperatures, with the NILs carrying *Eps*-late alleles showing slightly though consistently lower values (Fig. 6.8). More relevantly, the initial slopes were clearly different, indicating that the rate of development towards heading was more sensitive in the *Eps*-early (0. 807 10^{-3} [d ºC]$^{-1}$) than in the *Eps*-late NILs (0.720 10^{-3} [d ºC]$^{-1}$). This is the mechanistic bases for the delay produced by the *Eps*-late alleles in reaching anthesis. The reciprocal of the slope is the thermal time at the estimated base temperature, and this was c. 1240ºCd for the NILs carrying the *Eps*-early alleles, whilst it was 1390ºCd for the NILs carrying the *Eps*-late alleles. And in this particular case, the change in sensitivity was not only quantitative in nature, as it included an extreme situation (at 21ºC) in which 60% of the NILs carrying the *Eps*-early alleles continued developing normally until heading (though slower than at 18ºC) whilst all plants of the *Eps*-late NILs stopped developing towards heading.

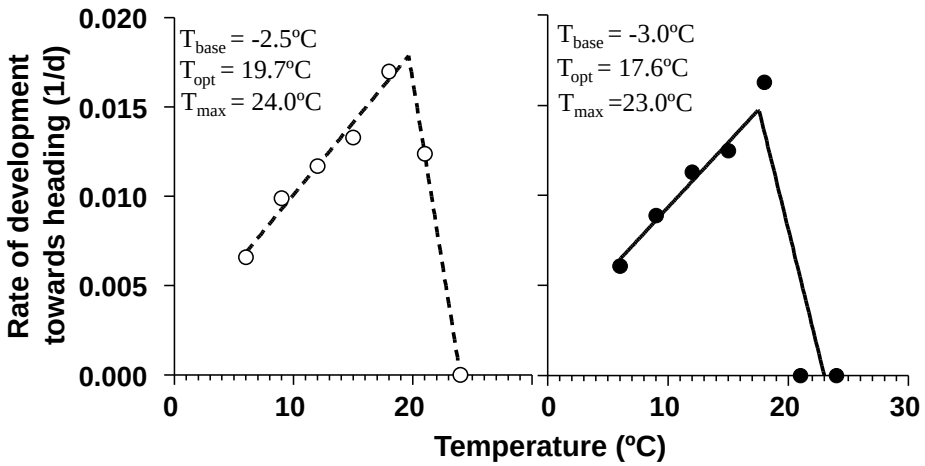

Fig. 6.8. Relationship between the rate of development between the onset of the experiment and heading and temperature for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 6, 9, 12, 15, 18, 21 and 24°C. Lines were fitted by bi-linear regression. Inset both panels are the calculated cardinal temperatures (base, T_{base}; optimum, T_{opt}; and maximum, T_{max}) for each group of NILs.

Regarding cardinal temperatures we found similar conclusions from analysing the rates of development during the two phases considered in this study as components of time to heading. In general they tended to be lower in the *Eps*-late than for *Eps*-early NILs; Fig. 6.9). Interestingly, the overall base temperature estimated when considering the whole period from the onset of the experiment to heading seemed to reflect a weighted average of the base temperatures corresponding to earlier and later developing phases, as the base temperature seemed to have increased noticeably from the early phase until terminal spikelet to the later phase following this stage (Fig. 6.9).

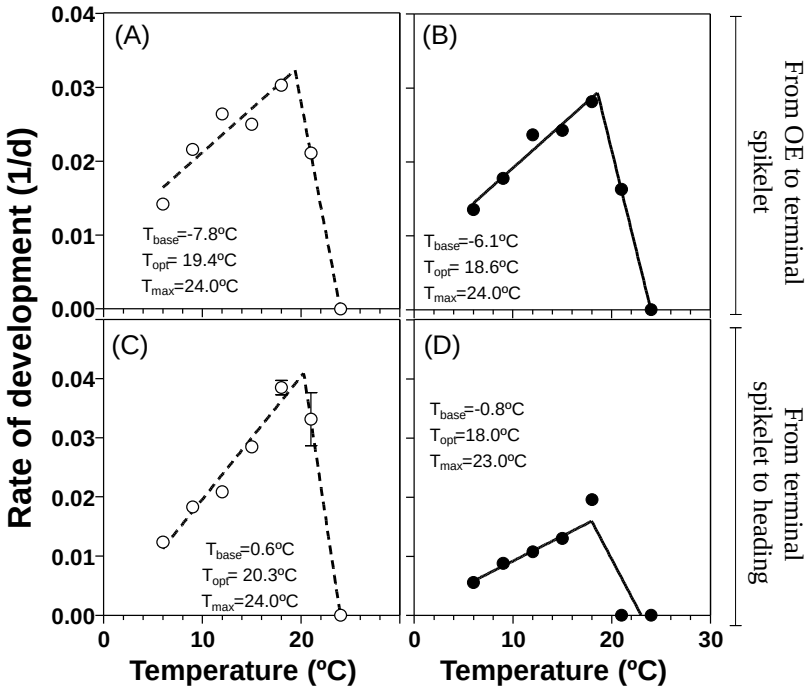

Fig. 6.9. Relationships between the rate of development between either from the onset of the experiment and terminal spikelet (A, B) or from then to heading (C, D) and temperature for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 6, 9, 12, 15, 18, 21 and 24°C. Lines were fitted by bi-linear regressions. Inset all panels are the calculated cardinal temperatures (base, T_{base}; optimum, T_{opt}; and maximum, T_{max}).

Importantly, the differences in slope were negligible for the phase from the onset of the experiment to terminal spikelet (c. 1.19 10^{-3} [d ºC]$^{-1}$, equivalent to c. 840ºC d for both *Eps*-late and -early NILs (Fig. 6.9A, B). On the other hand, the rate of development during the late reproductive phase was much more sensitive to temperature in the *Eps*-early (2.08 10^{-3} [d ºC]$^{-1}$, equivalent to c. 480ºC d; Fig. 6.9C) than in the *Eps*-late NILs (1.69 10^{-3} [d ºC]$^{-1}$, equivalent to c. 590ºC d; Fig. 6.9D).

We found the same pattern when analyzing the rates of either leaf appearance or spikelet primordia initiation and temperatures (Fig. S6.3). Rate of leaf appearance was relatively similarly sensitive to temperature in both the *Eps*-early and -late NILs (Fig. S6.3).

On the other hand, the rate of spikelet primordia initiation did show an effect of the *Eps*-alleles a difference between NILs: the rate of spikelet initiation of *Eps*-early NILs had a lower base temperature and, more relevantly, a higher sensitivity to temperature (0.072 vs 0.061 spikelets [d ºC]$^{-1}$) than that of the *Eps*-late NILs (Fig. S6.3).

6.4. Discussion

The fact that temperature, between the base and the optimum thresholds, hastened development was expected, as there is a huge body of literature reporting this effect in wheat development (e.g. Porter and Gawith, 1999; Slafer *et al*., 2015, and references quoted therein) as well as in other major crops (Parent and Tardieu, 2012), and even in other completely unrelated organisms (Gillooly *et al*., 2002; O'Connor *et al*., 2007). The overall delay in time to heading produced by the introgression of *Eps*-late alleles was also expected, as these lines were selected because of their previous reported effects in the field both in the UK (Zikhali *et al*., 2014) and in Spain (previous Chapter), where the actual effect of *Eps* alleles can be challenged, as they can be easily masked by the presence of other major developmental genes (Zikhali *et al*., 2014; Sukumaran *et al*., 2016). But what this paper contributes more relevantly is in providing direct evidence of the *Eps* x temperature interaction in hexaploid wheat. There have been already reports indicating the cultivars may differ in their sensitivity to temperature (e.g. Slafer and Rawson, 1995 in wheat, Karsai *et al*., 2013 in barley) as well as *Eps* x temperature interactions but working with an *Eps* gene of *T. monococcum* unusually strong (Bullrich *et al*., 2002; Appendino and Slafer, 2003; Lewis *et al.*, 2008); but to the best of our knowledge this is the first time this interaction is evidenced with an *Eps* gene that has the normal subtle effect on time to heading.

Indeed the magnitude of the deceleration of development produced by introgressing *Eps*-late alleles was stronger at relatively low than at mild and warm temperatures. The fact that the difference in time to reach heading between NILs carrying the *Eps*-late and -early alleles was affected by temperature was not due to a simple effect of temperature itself on the "extra time" required by the *Eps*-late NILs. In fact if we analyse the results in thermal time either using a general base temperature of 0ºC (Fig. 6.10A) or the estimated base temperatures in this study (Fig. 6.10B), the same interaction is still noticeable. Naturally the effect of temperature disappeared for the range 6-18ºC and the thermal time increases at 21ºC because this temperature was higher than the optimum.

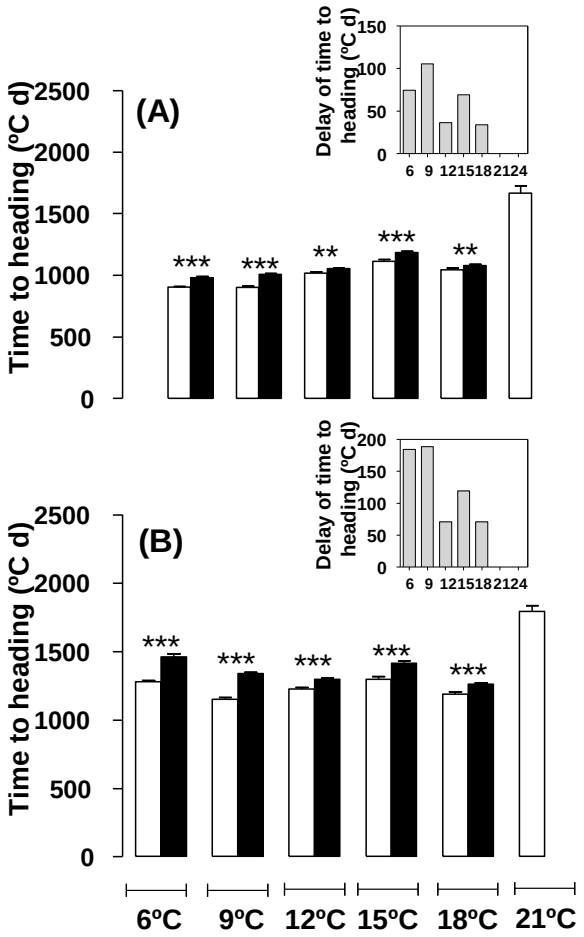

Fig. 6.10. Thermal time from the onset of the experiment to heading (estimated using 0ºC as base temperature, A; or the base temperatures estimated in Fig. 8) for lines carrying *Eps*- early (open bars) or *Eps*-late (closed bars) alleles grown under constant temperatures of 6, 9, 12, 15, 18, and 21ºC. Inset is a detail of the difference (in ºC d) between lines with the *Eps*-late or *Eps*-early alleles. Asterisks indicate the statistical significance of the differences between lines with *late* and *early* alleles at each temperature from the LSmeans contrast (***P<0.01*, ****P<0.001*).

This is in agreement with that we discussed in the Chapter V regarding the results presented in Zikhali *et al*. (2014) and our work with *Eps* under field conditions. When facing the fact that the effect of *Eps1D* were stronger in Zikhali *et al*. (2014) than in our work (Chapter V) we speculated that differences on ambient temperature were these NILs were grown in each case. In the present study we provided evidences that support that speculation. Results are naturally more clear when using the appropriately estimated base temperatures than when using 0°C for calculating thermal time, as a general base temperature may not be equally suitable (Slafer and Rawson 1995). Thus, it was demonstrated that the *Eps1D* gene studied in this work was likely a gene of sensitivity to temperature; and the *Eps*-early and –late allele determine different degrees of sensitivity. Furthermore, the fact that the sort of interaction we reported was not cross-over offers some confidence in that this allele could be an important

tool for wheat breeding when there may be interest in fine-tuning time to heading (at least it would not be expected reversals of expected results, as suggested may be the case with other *Eps* genes which might exhibit stronger interactions; Slafer, 1996).

It seemed clear that the reduction in time to heading produced by the introgression of the *Eps*-early allele from chromosome 1DL of Spark into that of Rialto was mainly associated with an increase in sensitivity to temperature during the late reproductive phase. We are not aware of other studies reporting these effects for *Eps* genes in hexaploid wheat, but the finding is consistent with the fact that different phases of a wheat and barley genotypes may respond to temperature with varying degrees of sensitivity (e.g. Slafer and Rawson, 1995, Karsai *et al*., 2013). This capacity of *Eps1D* to modify time to heading through modifying development during stem elongation is relevant as in most cases it is assumed that the effects of *Eps* genes are limited to (or at least are stronger in) the earlier phases of crop development (Slafer, 1996). Counting with alleles that affect the developmental rates of the late reproductive phase may be instrumental as they can be used not only to fine-tuning heading time but simultaneously the likely fertility of the spikes. Optimizing the duration of this developmental phase may be an avenue for further improving the likelihood of floret primordia to become fertile florets (e.g. Gonzalez-Navarro *et al*., 2015) which would further improve the number of grains (Slafer *et al*., 2001; Miralles and Slafer, 2007), which is the yield component most largely affecting yield (Slafer, 2003; Fischer, 2008).

The parallelism between the effects in total time to heading and in the late reproductive phase is commensurate with the finding that this *Eps* gene affected more consistently the period from flag leaf emergence to heading than the phyllochron; reinforcing that the effect on the duration of the late reproductive phase may well be concentrated in the last part of the stem elongation, when it might be even more critical for effecting grain number.

The interaction between *Eps* alleles and temperature on spikelet number per spike was not relevant, indicating that this yield component would not be affected when time to heading were fine-tuned with this *Eps1D* gene. This study contributed a better understanding the physiological and genetic determinants controlling the responses to different temperatures, providing new tools in front the changing temperatures caused by climate change.

6.5. References

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6.6. Supplementary material

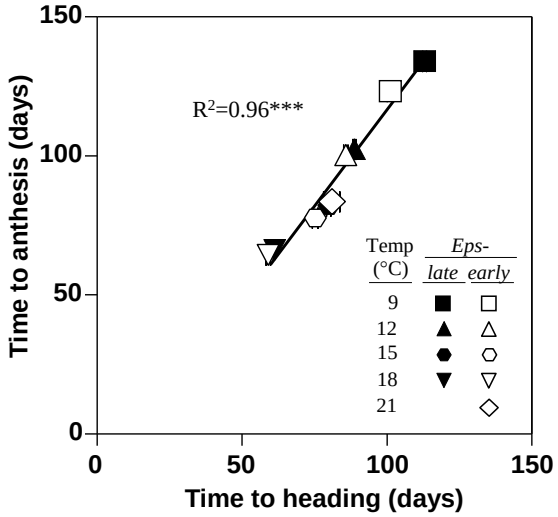

Fig. S6.1. Relationship between time to anthesis and time to heading for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 9, 12, 15, 18, and 21ºC (treatments at 6 and 24°C were not included as none of the plants at those conditions reached anthesis). Segments on each symbol stand for the SEM (if not seen is because the magnitude was smaller than the size of the symbol).

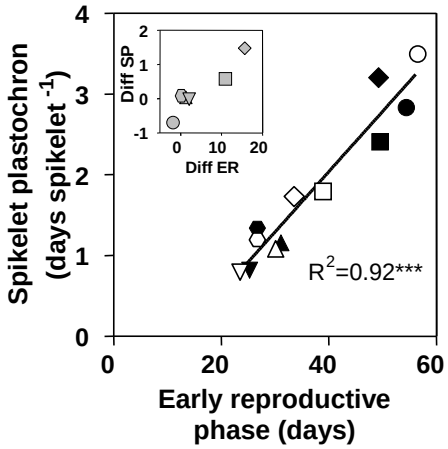

Fig. S6.2. Relationship between spikelet plastochron and the duration of the early reproductive phase for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 6 (circles), 9 (squares), 12 (triangles), 15 (hexagon), 18 (inverted triangles) and 21°C (diamond). Line fitted by linear regression (*P<0.001*). Inset is a detail of the relationship between the differences (*Eps*-late minus *Eps*-early lines) in spikelet plastochron (SP) and in duration of the early reproductive phase (ER).

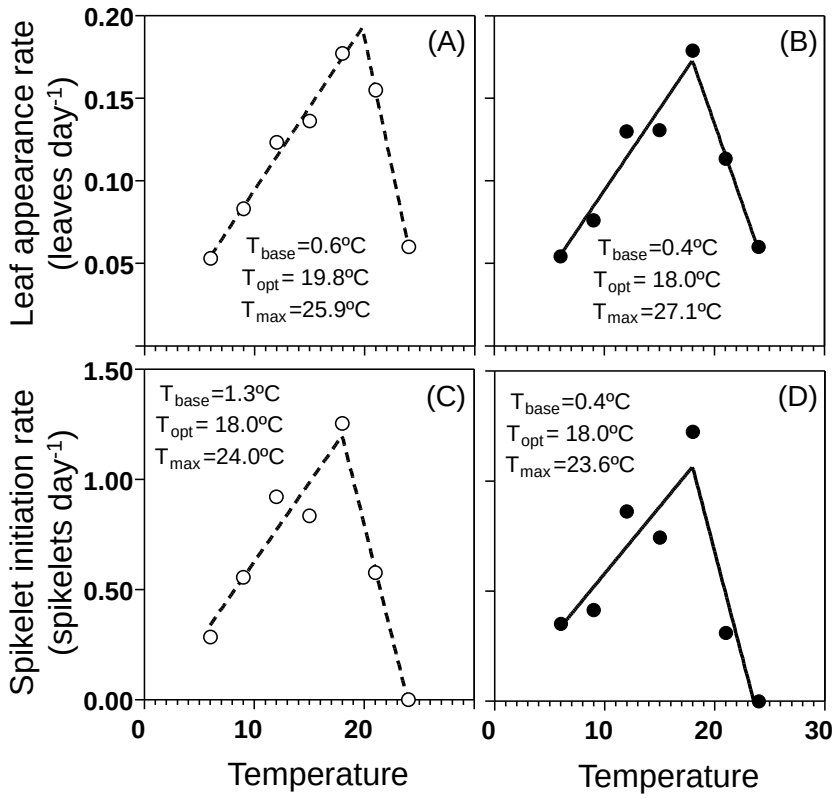

Fig. S6.3. Relationships between the rates of leaf appearance (A, B) or spikelet initiation (C, D) and temperature for lines carrying *Eps*-early (open symbols) or *Eps*-late (closed symbols) alleles grown under constant temperatures of 6, 9, 12, 15, 18, 21 and 24°C. For leaf appearance we used a weighted average of the rates corresponding to early- and late-appearing leaves. Lines were fitted by bi-linear regressions. Inset all panels are the calculated cardinal temperatures (base, T_{base}; optimum, T_{opt}; and maximum, T_{max}).

Chapter VII

Genetic variation in developmental attributes within modern and well adapted wheat cultivars

7. Chapter VII: Genetic variation in developmental attributes within modern and well adapted wheat cultivars

7.1. Introduction

Wheat provides *c*. 20% of the calories and proteins to the world´s population (Braun *et al*., 2010). In order to satisfy the expected global demand for wheat, yield should be improved at a rate of around 1.7% per year until 2050 (Rosegrant and Agcaoili, 2010). This takes into account the difficulties of increasing production significantly from further expanding the growing area (Albajes *et al*., 2013) implying that the required increase in demand must be satisfied by further increases in yield (Reynolds *et al*., 2012; Fischer *et al*., 2014; and references quoted therein). The required rates of yield gain contrasts with recent global trends in wheat productivity , evidencing rates of less than 1% y^{-1} (Reynolds *et al.*, 2012). Breeding for further raising yields would likely be the main route to increase productivity in a context in which the use of inputs would hardly increase further (Connor and Minguez, 2012). That is why breeding successfully to allow a rapid change in cultivars for better adapted and higher yielding ones seems critical in the near future (Atlin *et al.*, 2017).

When aiming to improve for yield or other complex traits, breeders are reluctant to include in their crosses anything that cannot be considered elite germplasm, trying to pyramid best combinable traits (Rattey *et al.*, 2009). In order to design strategic crosses, it seems sensible and valuable to quantify, within elite germplasm, the degree of genetic variation in relevant traits (Slafer, 2003; Foulkes *et al*., 2011; Reynolds *et al*., 2012). Among these traits are the developmental attributes responsible for adaptation (phenology) and generation of organs that will become sources and sinks of the crop (duration of vegetative and reproductive phases, and rates of initiation/appearance of vegetative and reproductive organs). Flowering time (key to adaptation and to determine yield) is delimited by three developmental phases: (i) vegetative, (ii) early reproductive and (iii) late reproductive. In these phases different organs are being generated and these organs will become sources-sinks determining yield (Slafer and Rawson, 1994).

Modern, well adapted, cultivars already have optimised their time to flowering. However, further improvements in yield might be possible by optimising the development patterns through changes on duration of each particular developmental phase composing flowering time (Slafer *et al*., 2001; Miralles and Slafer, 2007).

Therefore, quantifying the degree of variation in duration of different phases in modern cultivars would open opportunities for combining favourable allelic frequencies of these loci through breeding. And during the above mentioned phases in the apex are developing primordia, firstly of leaves and later of spikelets. As a consequence of this process, the variation on length of the different pheno-phases might be critical for the determination of the numbers of organs being initiated, provided compensations between rates of development and rates of initiation do not play a major role. A complementary way to understand flowering time can be decomposing it into the number of leaf primordia initiated in the apex, of the rate of leaf appearance (or its inverse, called phyllochron) and the period from flag leaf appearance and flowering (Jamieson *et al.*, 1998). These components may also be relevant to fine-tune phenology to improve yield. In addition, dynamics of primordia initiation may be coordinated with that of leaf appearance (Kirby, 1990) and identifying variation in this coordination may be relevant to modify phenology with varying degrees of compensations between duration of phases and number of organs initiated. The dynamics of leaf appearance may be also coordinated with tillering (Kirby *et al.*, 1985). As both, tillering and leaf appearance, are the responsible of capturing solar radiation that determine early growth and grain yield when the crop requires early vigour (Abeledo *et al.*, 2004), quantifying differences in phyllochron could also provide indirect indications on the dynamics of early tillering and in overall early vigour and therefore differences in phyllochron might be relevant for designing strategic crosses in regions where early vigour may be relevant.

Some studies explored the degree of variation on length of the developmental phase on elite germplasm that affected spike fertility traits (Elía *et al.*, 2016; Gonzalez-Navarro *et al.*, 2016), but, to the best of our knowledge, little is known about the degree of variation in the duration of the sub-phases, dynamics of primordia production and of leaf appearance.

We aimed to exploring to what degree modern and well adapted cultivars vary in detailed developmental attributes and whether they may be restricted in their use due to possible trade-off. More specifically, we quantified the genetic variation on

(i) time to heading and on the duration of vegetative, early reproductive and late reproductive phases,

(ii) final leaf number (FLN), phyllochron and duration of the flag leaf to heading time,

(iii) dynamics of leaf appearance, and (iv) dynamics of leaf and spikelet primordia initiation, determining the variation in leaf- and spikelet-plastochron on modern cultivars grown under field conditions in a Mediterranean region.

7.2. Materials and methods

7.2.1. Experimental set up

Two field experiments were conducted during 2012/13 and 2013/14 growing seasons nearby Bell-lloc d´Urgell (41.63°N, 0.78°E, 196 m above sea level) Catalonia, North East Spain, in a soil classified as a complex of *Calcisol petric* and *Calcisol haplic*, following the soil classification of FAO (1990). The experiments were sown on optimum dates for Mediterranean region of Spain (22 November 2012 and 12 November 2013) at a rate of 300 and 350 seeds m^{-2} for first and second growing season respectively). Experiments were irrigated when needed to avoid water stress. In both years, weeds, insects and diseases were prevented or controlled using commercial pesticides applied following the recommendations of manufacturers.

Daily global radiation, maximum and minimum temperature and accumulated precipitation were recorded daily in a meteorological station located close to the experiments. Although both growing seasons were rainier than the average of the previous (264, 303 and 246 mm, respectively), to avoid water stress we irrigated the fields around flowering (on April 2013, 26 March 2014 and 3 May 2014; each one of 80 l m^{-2}). Temperatures were in general similar in both growing seasons (in turn similar to the average of the previous 6 years; Fig. 7.1). Temperatures were similar in both seasons, with the exception that April was warmer in the second than in the first growing season (22.5 and 18.8ºC, respectively) (Fig. 7.1).

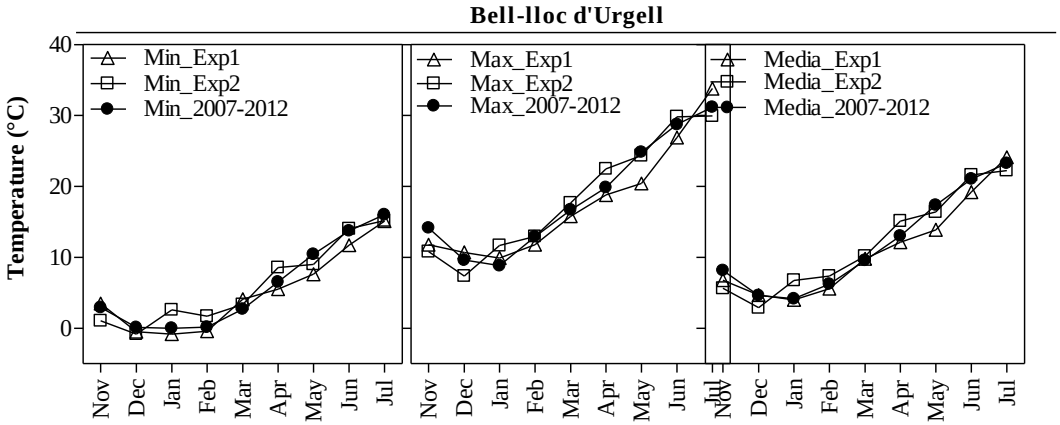

Fig. 7.1. Minimum (left panel), maximum (middle panel) and mean (right panel) temperatures averaged monthly in the first (Exp1) and second growing (Exp2) seasons and in the six years previous to the experiments (2007-2012).

7.2.2. Treatments and design

Treatments consisted in nine modern cultivars of hexaploid wheat (*Triticum aestivum L.*). Eight of them (Atae, Arthur Nick, Califa Sur, Garcia, Ingenio, Nogal, Sensas and Rodolfo) were commercial cultivars well adapted to this region (according with the information reported from GENVCE, a Group in charge of evaluation of new varieties of field crops in Spain; http://www.genvce.org/). The last genotype was a promising breeding line at the time of the experiments, which was later registered as a new cultivar under the name of Tribat I33.

Treatments were arranged in a randomized complete block design with three replications. Each experimental plot consisted of 6 rows o.20 m apart and 4 m long.

7.2.3. Measurements and analysis

Phenological stages were determined according to the decimal code developed by Zadoks *et al*. (1974). At seedling emergence, three plants per experimental unit were selected to be representative (same timing of seedling emergence to the mean of the population and in a plant density condition like the expected one) and labelled in order to monitor leaf appearance following the scale proposed by Haun (1973). From seedling emergence to anthesis, the number of leaves appeared were recorded frequently (from once to twice per week, depending on temperature). With these values the dynamics of leaf appearance (and estimates of phyllochron; i.e. thermal time elapsed between the appearance of two successive leaves) were analysed.

One representative plant per plot (three per treatment) was randomly sampled and taken to the lab, where plants were dissected under a binocular microscope (Leica MZ 7.5, Leica Microsystems, Heerbrugg, Switzerland) to (i) determine the developmental stage of apex according with Waddington *et al*. (1983) and (ii) count total number of leaf and spikelet primordia. With these records of the cumulative number leaf and spikelet primordia initiation the dynamics of initiation of priomordia was analysed (and leaf and spikelet plastochrons, thermal time elapsed between the initiation of two successive primordia in the apex, estimated).

Dynamics of leaf appearance and of primordia initiation were obtained by plotting the Haun stage and the number of (leaf and spikelet) primordia against thermal time. Whenever indicated by distribution of residuals either linear or bilinear regressions were fitted. For leaf appearance the relationship was reasonably linear whilst for primordia initiation the trends were unequivocally bi-linear with leaf primordia initiated much slower than spikelet primordia. Phyllochron was calculated as the reciprocal of the rate of leaf appearance. For primordia initiation, plastochron were obtained as inverse of the coefficient of regression of the relationship between initiated primordia and thermal time, reciprocal of the first slope was the leaf plastochron and that of the second slope was the spikelet plastochron. For the dynamics of primordia production the bilinear model was fitted with a fixed intercept (4 primordia, corresponding to the 4 leaves initiated during seed filling in the mother plant and therefore they already are developed in the embryo when the seeds are sown).

Time to floral initiation was estimated *a posteriori*, after FLN had been determined and double ridges and terminal spikelet stages were recorded based on the scale developed by Waddington *et al*. (1983).

Thermal time was calculated with mean air temperature (i.e. assuming for the calculations that a base temperature of 0 ºC is adequate and that maximum temperature was never above the optimum temperature).

For analysing the results we used a mix model, considering growing seasons, genotypes, and their interaction as fix factors, whilst blocks were nested within growing seasons and identified as random. Heritability was estimated using the variance components as follows: $h2 = \sigma 2g / [\sigma 2\ g + (\sigma 2\ ge/e) + (\sigma 2res/re)]$; where $\sigma 2g$ is the variance component of genotype; $\sigma 2ge$ is the variance component of the interaction genotype x environment; $\sigma 2res$ the error; e, the number of environments, and r, the number of replicates per trial.

Student's t test was used to test the significance among genotypes. All the analysis were

performed by JMP® Pro Version 12.0 (SAS Institute Inc. Cary, NC, USA).

7.3.Results

Considering time to heading as the most integrative trait analysed, it was clear that it was affected by both the growing season and the cultivars, but their interaction was negligible (comparing the mean squares of the interaction with those of the main factors) and not significant (Table 7.1). Consequently there was a strong consistency in time to heading among the cultivars, which resulted in a very high heritability (h^2=0.97).

When considering the final number of leaves and spikelets produced, the situation was virtually the same for the former: FLN was not significantly affected by the interaction and therefore the genotypic differences were consistent across years (h^2=0.74). But in the case of the number of spikelets per spike, the interaction was significant, but even in this case the magnitude of the mean square of the interactions was much lower than the mean squares of the genotypes (Table 7.1). Consequently even when the interaction was significant it was not very relevant agronomically as the cultivars analysed exhibited a rather high heritability for spikelets per spike (h^2=0.92).

Table 7.1. Mean squares of the effects of the growing season (S), the genotypes (G) and their interaction (GxS) on the main traits analysed: (i) thermal time from sowing to heading, (ii) final leaf number and (iii) spikelet number.

Source of variation	Sowing to heading (°C d)	Final leaf number (leaves)	Number of spikelets (spike^{-1})
Season (S)	539,480***	4.75***	2.51 ns
Genotypes(G)	10,252***	1.29**	10.09***
G x S	269 ns	0.33 ns	0.76***

Asterisks indicate the significance level of the F-ratio (**P<0.05*, ***P<0.01*, ****P<0.001*, ns-non significant)

Therefore, for simplicity the results are presented focused on the genotypic variation (which has been the aim of the work) after data have been averaged across the two growing seasons.

7.3.1 Phenology

Thermal time to heading was relatively similar among cultivars, ranging from c. 1040 to 1143°C d, as expected when the source of variation are modern cultivars which are all well adapted. Atae had the shortest period from sowing to heading, and this duration was not significantly different to that of Califa Sur and Arthur Nick (Fig. 7.2). On the other extreme of the range, Garcia and Tribat I33 had the longest period (Fig. 7.2).

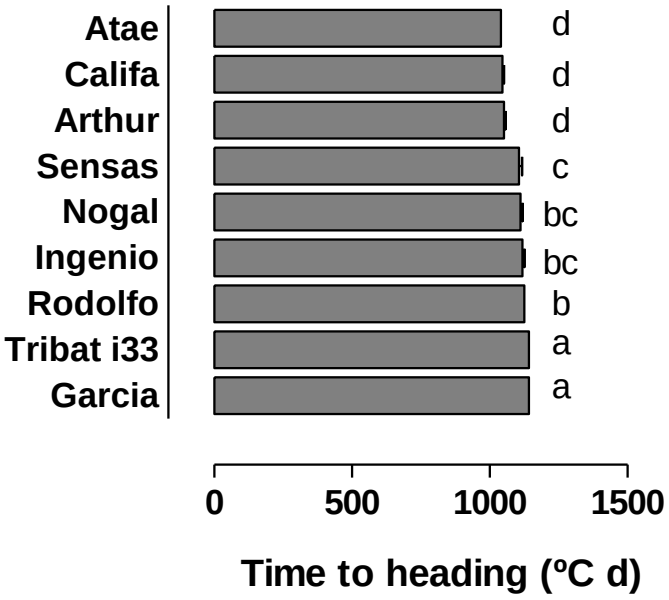

Fig. 7.2. Thermal time to heading for the nine modern cultivars. Different letters indicate that cultivars were significantly different on heading time.

Disregarding the fact that there was relatively little variation in time to heading, there seemed to be more clear differences, in relative terms, in the duration of the phases that compose time to heading. The duration of the vegetative phase was intrinsically short but exhibited some degree of variation (Fig. 7.3A). On the other hand, the duration of the early reproductive phase, even though intrinsically longer than the previous one, did not show significant differences among the cultivars (Fig. 7.3B). The late reproductive phase was intrinsically the longest and did also show genotypic variation (Fig. 7.3C).

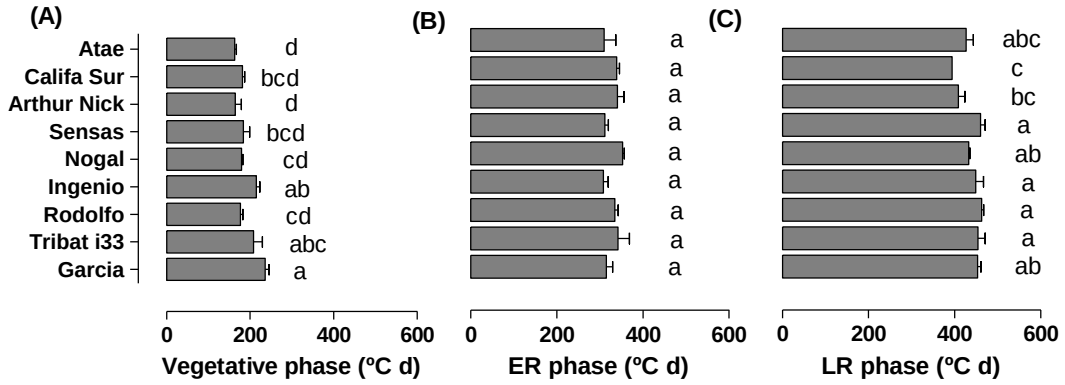

Fig. 7.3. Duration (in thermal time) of the vegetative phase (from emergence to floral initiation; A), the early reproductive phase (from floral initiation to terminal spikelet; B), and the late reproductive phase (from terminal spikelet to heading; C) for nine modern cultivars. Different letters indicate that cultivars were significantly different.

Interestingly there was no apparent relationship between the variations in the different phases: it could be possible to identify lines with different combinations of durations of different phases (Fig. 7.3). For instance, Atae and Garcia were the cultivars with the shortest and the longest vegetative phases but they did not differ significantly in duration of the late reproductive phase, or contrarily when comparing Califa Sur and Rodolfo, they did not differ in the duration of the vegetative phase but had differences in the duration of the late reproductive phase. This implies that a certain time to heading may be reflecting different partitioning of the developmental time between different phases. And that there may be no relationship between the duration of one phase and the other across cultivars (e.g. a cultivar may have genetic factors accelerating development in one phase but slowing it down in the following phase, as illustrated by the lack of relationship between duration of phases from sowing to terminal spikelet and from then to heading; Fig. 7.4).

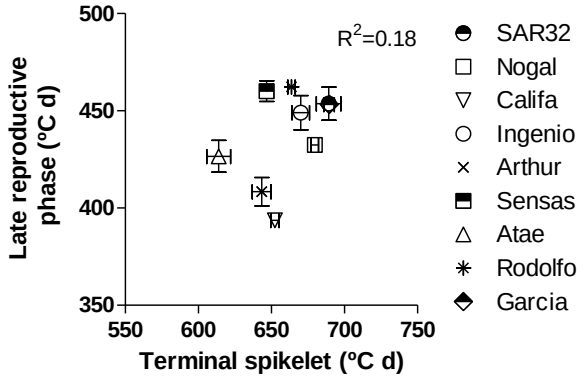

Fig. 7.4. Relationship between the duration of the late reproductive phase from terminal spikelet to heading and that from sowing to terminal spikelet for nine modern cultivars.

When considering the relative importance of the different phases for determining variation in time to heading across all cultivars it seemed that the variation in the late reproductive phase was the critical one (Fig. 7.5). Although the relationship of time to heading with the duration of the vegetative phase was statistically significant as well, the variation of the vegetative phase explained less and the magnitudes of changes in this phase were too small compared with those in time to heading (Fig. 7.5).

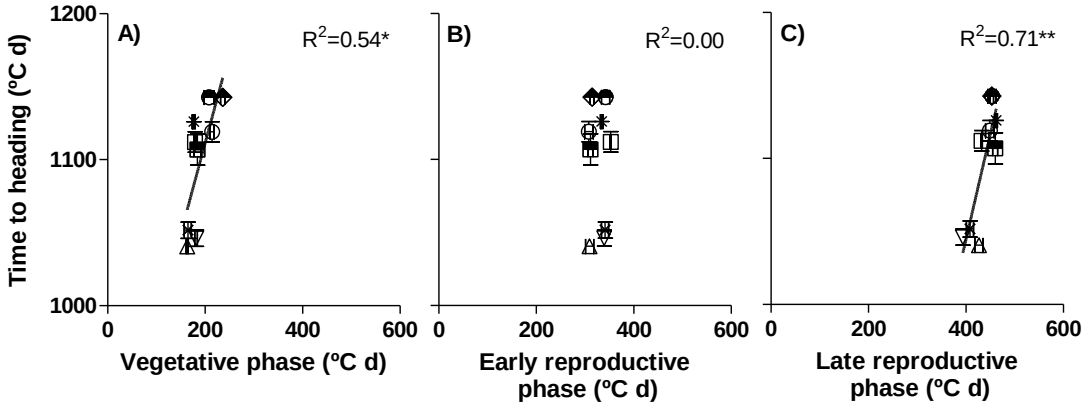

Fig. 7.5. Relationship between thermal time from sowing to heading and different developmental phases (in thermal time) for nine elite genotypes. The coefficients of determination (R^2) and the levels of significance (***P < 0.01,*P < 0.05*) were included.

7.3.2. Leaf number

Although in some cases a bilinear relationship could likely be more adequate, the relationship between the cumulative number of leaves and thermal time was strongly linear in all cases (Fig. 7.6). In all cases the coefficients of determination for the linear relationships were extremely high (R^2>0.975; *P<0.001*).

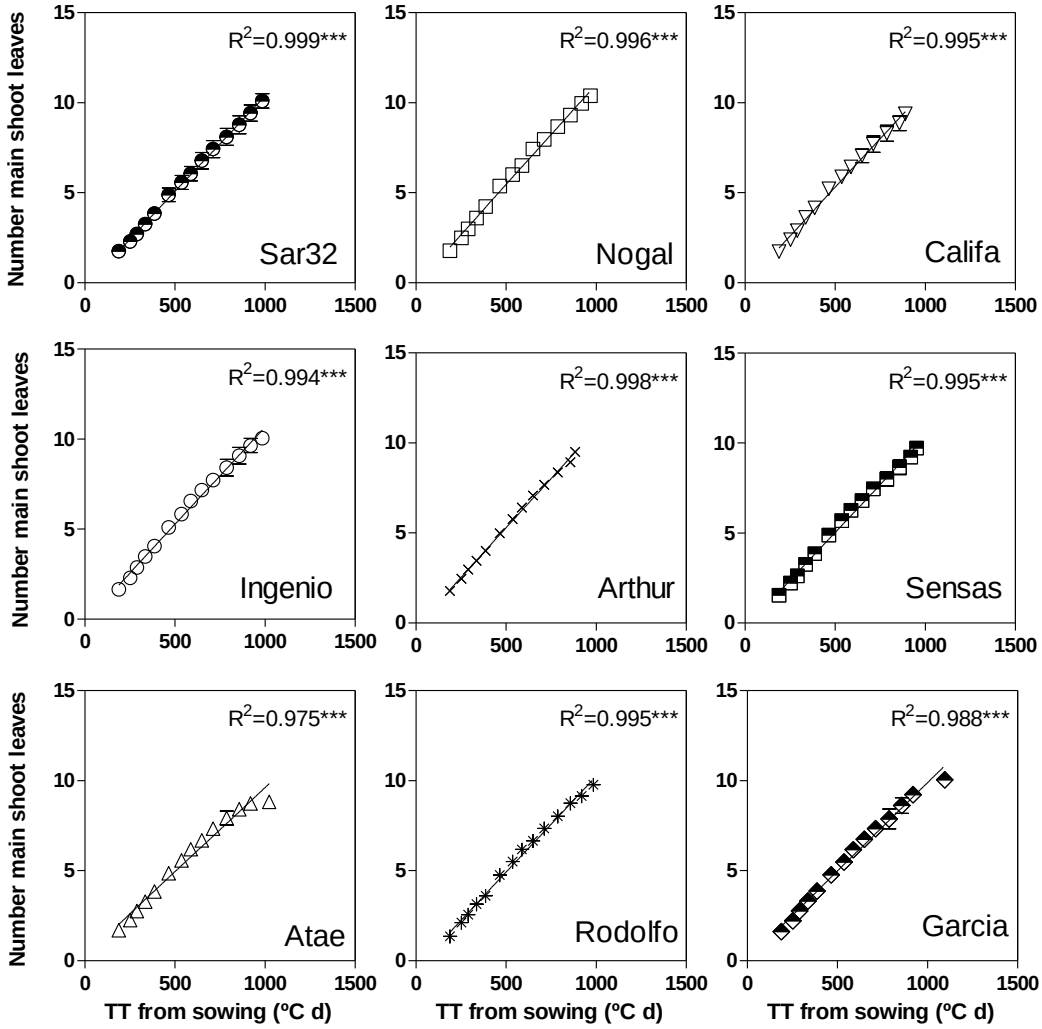

Fig.7.6. Relationship between number of emerged leaves and thermal time for nine elite genotypes.Line was fitted with linear regression. The coefficient of determination (R^2) and the level of significance (****P < 0.001*) were included.

Cultivars differed in the rates of leaf appearance and as a consequence they showed differences in phyllochron, as well as in final number of leaves (Table 7.2). Again it seemed that these two variables were not tightly linked and that it would be possible to combine in a single genotype different phyllochrons regardless of the number of leaves it may have. For instance, Garcia and Ingenio had exactly the same FLN but they differed in phyllochron (Table 7.2).

Table 7.2. Phyllochron and final leaf number (FLN) for nine elite genotypes averaged across growing seasons.

Genotypes	Phyllochron (°C d leaf)	FLN (leaves)
Atae	106.8±4.8 a	8.8±0.2 d
Califa Sur	92.5±2.0 b	9.4±0.1 c

Arthur Nick	91.0±1.5 b	9.5±0.2 c
Sensas	94.3±1.9 b	9.7±0.1 bc
Nogal	89.8±1.7 b	10.4±0.1 a
Ingenio	92.5±2.2 b	10.1±0.1 ab
Rodolfo	93.6±1.9 b	9.8±0.1 bc
Tribat I33	94.3±1.0 b	10.1±0.4 ab
Garcia	101.7±3.2 a	10.1±0.1ab

Different letters indicate that cultivars were significantly different for studied traits *Students* t analysis.

But when considering the variation across all cultivars, it seemed clear that the variation in time to heading was due to variation in FLN, whilst differences in phyllochron were completely unrelated with time to heading (Fig. 7.7).

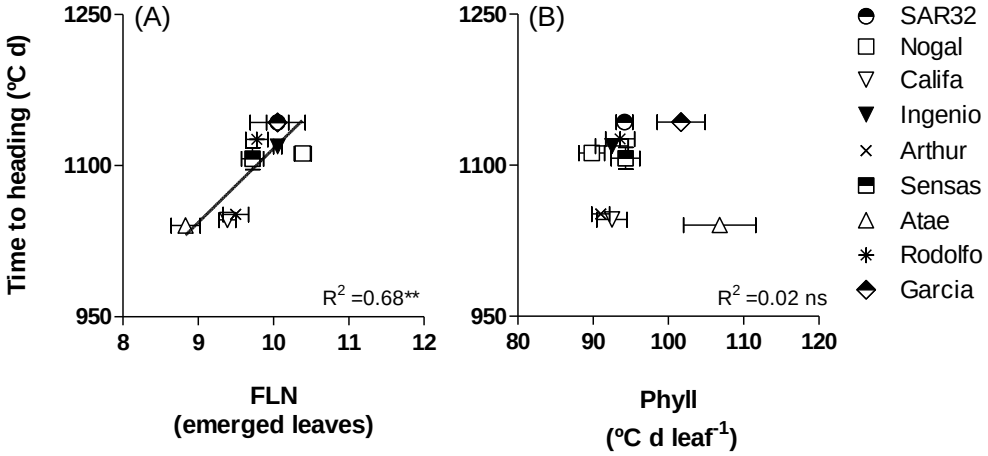

Fig. 7.7. Relationship between time to heading and either the number of main shoot leaves (A), or phyllochron (B) for nine elite genotypes. The coefficient of determination (R^2) and the level of significance (***P < 0.01*) were included. Line in panel A was fitted with linear regression.

There could be seen as a paradox the fact that time to heading was better related to the duration of the late reproductive phase as well as to FLN, when all leaves are initiated in the vegetative phase. But the link between both relationships is that when the FLN increases it also increases the number of leaves that must appear during the late reproductive phase, and there was a strong relationship between these two traits (Fig. S7.1).

7.3.3. Leaf and spikelet plastochron

Expectedly, as it is well documented that leaf initiation is slower than spikelet initiation, in all cases the relationship between the cumulative number of apex primordia and

thermal time was strongly bi-linear (Fig. 7.8), with very high coefficients of determination (R^2>0.967; *P<0.001*).

Consequently leaf-plastochron was substantially longer than spielet-plastochron (Table 7.3). Averaging across cultivars, leaf-plastochron was almost thrice as long as spikelet-plastochron (Table 7.3).

There were only minor differences among cultivars in leaf-plastochron (Table 7.3) suggesting that differences in FLN reflected those in the duration of the vegetative phase.

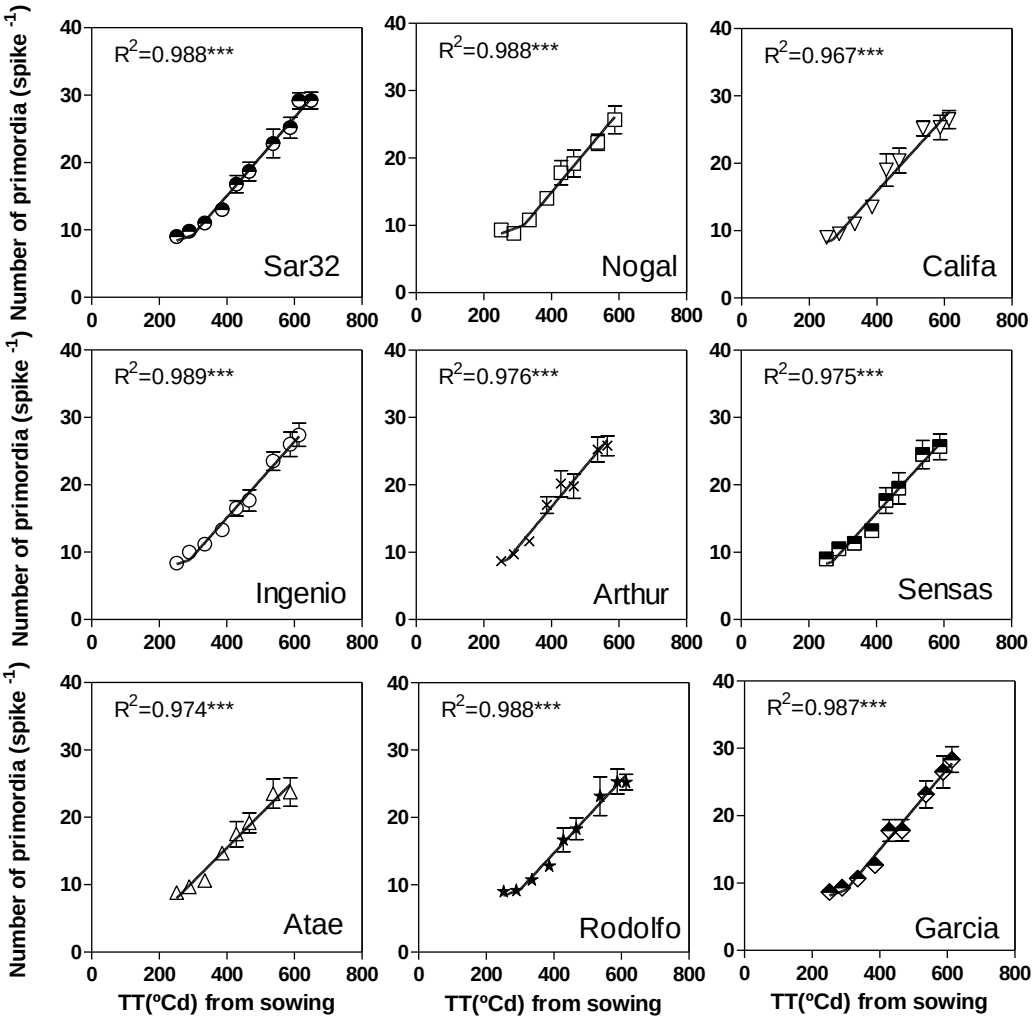

Fig.7.8. Relationship between number of apex primordia and thermal time for nine elite genotypes. Lines were fitted with bi-linear regression. The coefficient of determination (R^2) and the level of significance (****P < 0.001*) were included.

On the other hand, spikelet plastochron did show differences among modern cultivars, ranging from 16.5 to almost 20ºC d spikelet^{-1} (Table 7.3).

The variation in the rate of spikelet initiation brought about variations in number of

spikelets per spike (Table 7.3), which could not be explained by changes in duration of spikelet initiation. Spikelets per spike number varied between 16.2 for Rodolfo and 20.5 for Garcia. The differences in duration of the early reproductive phase were not related to those in either spikelet plastochron (Fig. S7.2A) or spikelet number (Fig S7.2B). Consequently, differences in the rate of spikelet initiation were relevant in determining genotypic variability in the number of spikelets initiated by each of the modern cultivars: there was a negative relationship between the number of spikelets per spike and spikelet plastochron (Fig. S7.2C). In other words, genotypic variation in the rates of spikelet initiation determining variation in spikelet plastochron were instrumental in generating variation in number of spikelets per spike.

Table 7.3. Leaf plastochron (ºC d leaf^{-1}), spikelet plastochron (ºC d spikelet^{-1}) and number of spikelets per spike for nine elite genotypes averaged across growing seasons.

Genotypes	Leaf plastochron (ºC d leaf^{-1})	Spikelet plastochron (ºC d spikelet^{-1})	Number of spikelets spike^{-1}
Atae	63.0±1.3 a	19.9±1.3 a	16.6±0.2 b
Califa Sur	58.1±1.4 a	18.1±1.2 a	18.9±0.1 b
Arthur Nick	55.0±1.2 a	16.7±1.1 b	18.7±0.2 b
Sensas	58.7±1.0 a	18.2±1.1 a	18.6±0.1 bc
Nogal	51.2±1.3 a	17.1±1.6 a	17.5±0.1 d
Ingenio	59.8±0.8 a	17.7±0.9 a	18.1±0.1 c
Rodolfo	57.0±0.8 a	18.4±0.9 a	16.2±0.1 e
Tribat I33	57.3±0.6 a	17.2±0.6 a	18.9±0.3 b
Garcia	61.9±0.6 a	16.5±0.6 b	20.5±0.2 a

Different letters indicate that cultivars were significantly different for studied traits *Students* t analysis.

7.4. Discussion

As we expected, the range of time to heading was relatively narrow as we did not attempted to uncover the degree of variation across the species, but proposed to find out genotypic variation in developmental attributes within modern, well adapted, cultivars. By definition, if all were well adapted, their differences in time to flowering must be small, as flowering time is the most critical trait determining adaptation in crops (Cockram *et al*., 2007). Thus the aim was not to uncover the degree of variation in time to flowering, but to study what subtle variation in other developmental traits might be hidden behind similar times to heading. And this is relevant in practical use of the information as breeders restrict their crosses to elite x elite materials when attempting to pyramid yield gains (Rattey *et al*., 2009). Also as expected, we found out a rather high

level of heritability (which does actually estimate better the "repeatability" of differences among cultivars across growing seasons than actual heritability of the trait), otherwise the concept of "well adapted", in terms of adequate timing of heading, would not have sense. It simply confirmed that it is a trait with a strong genetic control given mainly by different sensitivity to photoperiod and vernalization and to a lesser extent due to differences in earliness *per se*. This genetic control of time to heading has been widely documented in the literature (Baum *et al.*, 2003; Hanocq *et al.*, 2007; Cockram *et al*., 2007; Griffiths *et al.*, 2009, just to mention a few). But as largest variations in time to heading are given mainly by *Ppd* and *Vrn* genes it would not be expected to be strongly affected by GxE interactions when the "E" are growing seasons in the same region and within similar sowing dates (definitively the GxE could be huge if the genotypes differ in *Ppd* or –mainly- *Vrn* genes and the "E" are sowings in different regions or deeply different sowing dates; which also perfectly complies with the concept of adaptation).

Understanding the genetic variation of partitioning of time to heading time into three developmental phases may be a useful tool to breeders in order to explore variation of the duration of the phases and consequently the possible variation in the number of the formed organs (depending on the quantification of likely trade-offs) which may potentially contribute to yield. In our work, the variation on heading presented on the nine Spanish genotypes was explained mainly by changes of the length of both vegetative and late reproductive phases. Changes caused on the duration from emergence to floral initiation seemed to be independent of changes caused from terminal spikelet to heading time, these results agreed with those from other researches (Halloran and Pennel, 1982, González *et al.*, 2002; Whitechurch *et al.*, 2007; García *et al.*, 2011). This independent variation in duration of different phases of time to heading shows that it is possible the manipulation of duration of pre-flowering phases without modifying the whole period of heading time (Borràs *et al.*, 2009). Thus, these findings might be considered as potential sources to produce new varieties with improved yielding capacity, as the balance in duration of different phases has been suggested as a trait that may influence yield, in the absence of changes in the overall period to heading (Miralles and Slafer, 2007).

When it was analysed the others components determining time to heading time, i.e, FLN and phyllochron, it was reported variation on time to heading may be associated to changes on FLN without compensatory effects on phyllochron, in line with previous

studies (e.g. Whitechurch and Slafer, 2002; González *et al.*, 2005). These results may be interpreted as if variations in duration of the whole period from sowing to heading would affect the early developmental stages, when leaves are initiated; which in turn might affect early vigour, a trait that may improve water-use efficiency and grain yield in some regions (Duan *et al.*, 2016). However, this is not necessarily true, as the higher the FLN the higher the number of leaves that must appear during the late reproductive phase and therefore disregarding the fact that leaves are initiated in the vegetative phase, an increase in FLN was associated with a lengthening of the later phases. This in turn is due to the fact that leaf plastochron is shorter than phyllochron, and therefore the higher the FLN the more leaf primordia are "queuing" to appear later, lengthening the late reproductive phase. Furthermore, the existence of trade-offs between duration of a phase and the rate of primordia initiation during that phase may well imply that a shorter phase of leaf and spikelet initiation could not necessarily determine reductions in the number of leaves and spikelets. In addition, even reducing the earlier phases may not directly imply a loose in early vigour as changes in phyllochron (and tillering) may be independent of those in rate of development for the phase (e.g. Borràs *et al*., 2009).

In conclusion, we found that even within modern cultivars all well adapted there was minor variation in heading date which relevantly was mainly associated with variation in the duration of the late reproductive phase, due to the differences in FLN determining changes in the number of leaves to appear in later phases as a consequence of the difference between plastochron and phyllochron. Variation in the duration of the early reproductive phase was minor and unrelated to the rate of spikelet initiation. Thus, variation in spikelet plastochron determined the variation among the cultivars in the number of spikelets per spike. Then, spikelet number was not affected by variation in heading time or its partitioning into developmental phases.

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7.6. Supplementary material

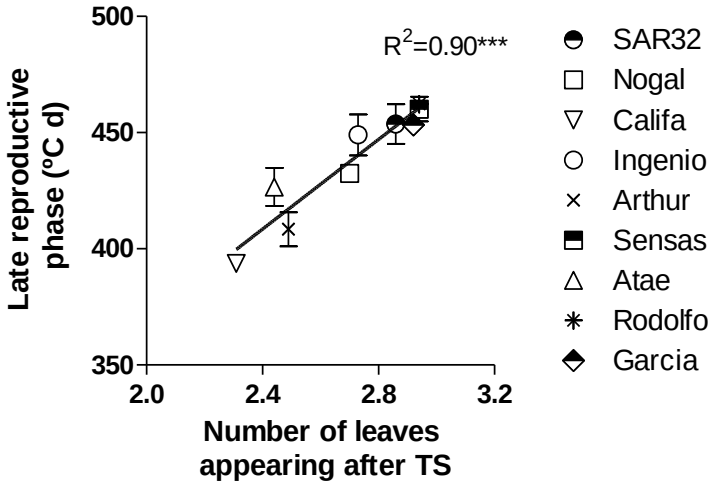


Fig. S7.1. Relationship between the duration of the late reproductive phase and the number of leaves that must appear from terminal spikelet onwards. Line was fitted with linear regression.

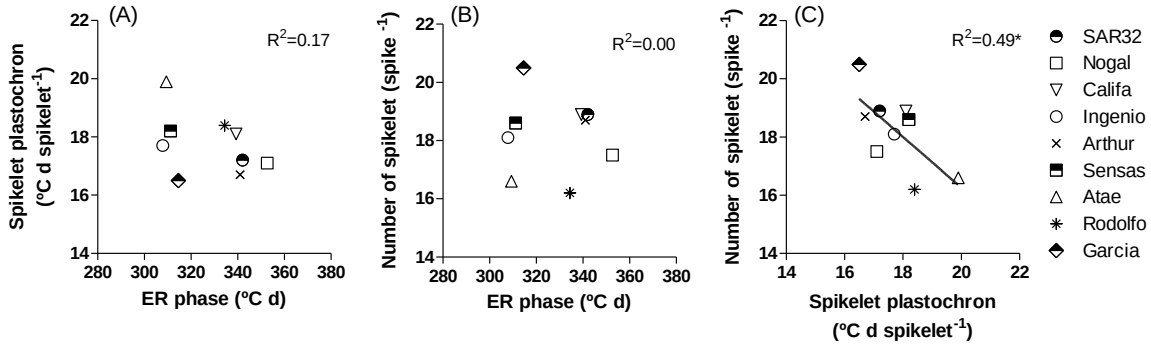


Fig. S7.2. Relationship between the duration of the early reproductive phase and spikelet plastochron (A), number of spikelets per spike (B) and the relationship between number of spikelets and spikelet plastochron (C). Line was fitted with linear regression.

Chapter VIII

General discussion

8. Chapter VIII: General discussion

8.1 Why this research? Brief context.

Phenology is, beyond any questioning, the most critical individual trait that may contribute to the adaptation of wheat (and any other field crop) to new environments, including those being created by climate change. Due to the complex genetic control (with at least three different groups of genes: those determining photoperiod sensitivity, vernalisation sensitivity, and earliness *per se*), wheat phenology has the potential to be adapted to most conditions projected for the future climate (as it has been adapted to virtually all agroecosystems of the world). However, to deploy through breeding programmes the variation produced by this complex genetic control more efficiently we must reach an appropriate characterization of alleles/allelic combinations responsible for particular developmental traits.

Although there is some empirical knowledge of genotypic differences in heading time (e.g. Slafer and Rawson, 1994), and its value for adaptation (Araus *et al*., 2002), the physiological and genetic bases of these differences are relatively well characterised for the overarching developmental trait of time to heading/anthesis, but when it goes to components of this general trait (being these components either duration of particular sub-phases or the combination of final leaf number and phyllochron) the knowledge is much less (and much less clear), and if it goes to the development of individual organs taking place during the specific sub-phases these bases are largely not known. Likewise, the major effects of temperature and photoperiod on time to heading/anthesis are well described but the effects of these environmental factors on its physiological determinants and on the dynamics of organogenesis are far less described and only fragmentarily known. The above statement is true not only for photoperiod and vernalisation sensitivity genes but also, and particularly so, for earliness *per se* genes.

Thus, although a large body of work on wheat phenology is available in the literature, there are still several issues that needed further analysis and were part of the studies carried out in this Thesis:

(i) genetic factors controlling time to flowering were well established in terms of genes that are responsive to photoperiod and vernalisation (Yan *et al*., 2004; Turner *et al*., 2005; Beales *et al*., 2007; Wilhelm *et al*., 2009), and more vaguely described for some earliness *per se* (Griffiths *et al*., 2009; Hancoq *et al*. 2007; Lewis *et al*., 2008), but

specific relevance of them when acting in a particular genome, for coarse- and fine-tuning time to anthesis has not been widely determined;

(ii) differences in sub-phases constituting time to heading have been shown to exist (e.g. Whitechurch *et al*., 2007) but have been only superficially analysed so far, even when they may potentially become a critical physiological tool for further increasing in wheat yield (Slafer *et al*., 2001);

iii) genetic factors controlling partitioning of developmental time to anthesis into different phases have shown inconsistencies (e.g. Gonzalez *et al*., 2005), and knowledge of those controlling physiological determinants of this phenological partitioning is in its infancy. A more comprehensive and quantitative understanding of the physiological and genetic determinants of time to heading and partitioning of developmental time into particular phenological phases of pre-flowering would open opportunities for the optimisation of developmental patterns for maximising yield;

(iv) studies of the differences in leaf number and phyllochron and their interrelationship have been mostly descriptive in response to environmental factors (e.g. Miralles *et al*., 2001; Garcia del Moral *et al*., 2002), but the effects of particular genes on the dynamics of leaf appearance were only fragmentarily described (e.g. Borràs *et al*., 2009; Sanna *et al*., 2014);

(v) research works quantifying dynamics of primordia initiation have been scarce in general due to the difficulties in collecting the microscopic data, and very few of them have attempted to relate them with the dynamics of other developmental processes, which would be relevant to ascertain whether the factor under study (environmental or genetic) affect leaf- and spikelet-plastochron directly or simply reflecting the overall effects of the factor on crop development (which in turn is useful to understand the existence, or lack, of compensations in final number of organs).

Trying to shed light in all preceding points, in this Thesis, I quantified for them the effect of introgressing *Ppd-1a* alleles (into a common background considering which genome (A, B and/or D), how many genomes carrying the mutation and the donor for one of them), and *Eps* alleles and explored the degree of variation among modern wheats.

In this chapter, I have mainly focused in integrating the main results across different experimental chapters, summarising the outcomes of testing of the main hypothesis and finally highlighting the contributions of the knowledge achieved.

8.2. Integration of the main results

A major aim across the different experimental chapters was to assess to what degree the genetic variation in flowering time (key to adaptation) was associated with variation in its components, i.e. to determine if the effects of particular *Ppd* or *Eps* genes, or if the differences among elite germplasm, on time to flowering were due to variation in (i) the durations of particular pre-flowering developmental phases (vegetative, early and late reproductive phases); and (ii) final leaf number (FLN) or phyllochrons.

Regarding the effects on / differences in duration of particular phases, changes in flowering time were found to be associated with those in the length of all three phases analyzed. When analyzing the whole data set of the different treatments across all the experiments, variation in duration of all phases was similarly relevant in determining differences in time to flowering across all the different sources of variation of this Thesis (Fig. 8.1A). However, these overall relationships have two distinct groups of sources of variations: the different experiments in which the different genetic materials were grown and the genetic differences within experiments. And a major driving force behind the overall relationships across the Thesis was the difference among experiments: time to flowering and the length of each of the phases considered were longest in the experiments with the *Eps* NILs, shortest in the experiments with the set of modern cultivars and intermediate in those in which *Ppd* NILs. In order to disregard the differences caused by the experiments, getting a fair appreciation of the overall genetic effects on duration of different phases as components of time to flowering, we transformed the data within each of the sets of genotypic treatments as residuals of the mean for the group of treatments within experiments (i.e. estimated the value of each developmental trait with respect to the average of all the genotypes of each particular experiment). Thus, excluding the "experiment" effect, it can be seen that overall genotypic differences in time to flowering seemed to have been more consistently related to their difference in length of both the vegetative and late reproductive phase rather than in that of the early reproductive phase (Fig. 8.1B).

If the effects of genotypes within particular experiments are analyzed in more detail and separately, the main phases being sensitive change with the particular genotypes experimented. When considering photoperiod insensitivity genes, *Ppd-1a* alleles accelerated development towards anthesis more through accelerating the early reproductive phase than the other two phases considered (Fig 3.4, left panel, Chapter

III). However, when *Eps1D* NILs and set of modern cultivars were considered, the variation in total time to flowering time was mainly due to changes on the length of late reproductive phase (Fig. 5.4, Chapter V; and Fig. 7.5, Chapter VII, respectively).

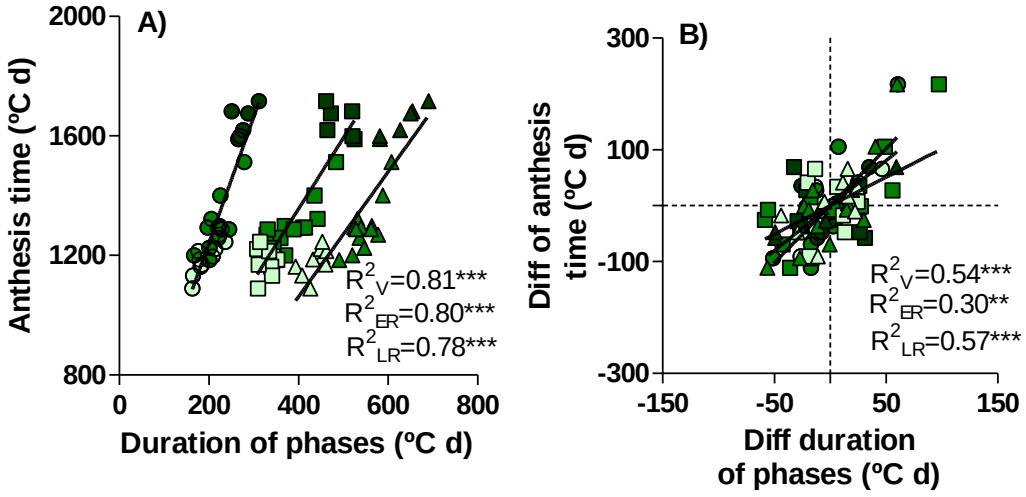


Fig.8.1. Relationship between time from sowing to anthesis and the duration of the three component phases (vegetative -circles- early reproductive-squares-and late reproductive-triangles-) for both *Ppd* NILs+ wild type (Paragon)-dark symbols- and *Eps* NILs-intermediate colours symbols-, and all modern cultivars- light symbols- (A). Relationship between differences on time to anthesis and differences on the sub-phases calculated as residuals of the average of all treatments within each experiment The coefficient of determination (R^2) and the level of significance (***P>0.01* and ****P<0.001*) were included.

Furthermore, in some cases (e.g. Fig 3.4, right panel of Chapter III), there was different partitioning of phases with similar time to anthesis which indicate the possibility of manipulating the length of each phase separately, as it was suggested previously (Halloran and Pennell, 1982; Miralles and Richards, 2000; Whitechurch *et al*., 2007). Therefore, within the lines used in this Thesis, there is a source of variation that may be useful for breeding programs. As we could observed different patterns of development depending on the number of *Ppd-1a* alleles introgressed or the donors of these alleles and on *Eps* alleles studied or their donors or the chromosomes where they were introgressed.

One focus of interest in the research of this Thesis was to explore to what degree reductions of the length of developmental phases by the genetic variation studied affected the number of organs generated. In other words, whether adjustments in duration of phases might produce parallel changes in number of organs or whether there

could be compensations through possible direct effects of the genetic variation considered on the rates of primordia initiation. Any differences in leaf number might be potentially compensated through crop management by modifying plant density in opposite directions (if the genetic change in adaptation reduced the number of leaves a more dense crop might compensate this reduction in terms of radiation interception). On the other hand, reductions in spikelets per spike could potentially affect yield potential and differences in spikelets per spike would be hardly compensated by crop density (which might for instance compensate for spikes per plant). Thus in this thesis a major interest was to establish the existence (or lack of) compensations in spikelets per spike through identifying genes modifying similarly the rate of development during the early reproductive phase and the rate of spikelet initiation. In order to assess if the shortening in the early reproductive phase duration similarly affected the number of spikelets, the duration of early reproductive phase was plotted against number of spikelets for all data across each experimental Chapter (Fig.8.2). A linear regression and significant R^2= 0.48, *P<0.0001* was found (Fig.8.2A). But, it is important to note that (i) even considering the overall effects across the whole dataset collected, the range of variation for early reproductive phase was higher (1.70 in relative values) than the number of spikelets (1.45 in relative values), indicating that a reduction of early reproductive phase was at least partially compensated with effects on the rate of spikelet initiation resulting in relatively modest changes in spikelets per spike; and (ii) that again a major driving force for the positive relationship was the difference between experiments. Redrawing the figure with the residuals of the means within each experiment, and therefore excluding the effect caused by experiments, the number of spikelet was not significantly related to the length of the early reproductive phase (Fig.8.2B). This compensation between both rates was true for the all the sets together, but considering individual experiments it was true for the overall effects of *Eps* genes and for the differences between modern genotypes, whilst when considering the situation for the *Ppd* NILs there was still a positive relationship –which would imply that the shorter the phase the less spikelets would be initiated- but the effect was strongly driven by the wild type Paragon, the data-point on the top-right of the cloud (Fig. 8.2B). Thus, in general there seemed that it was possible to reduce the duration of the early reproductive phase with a concomitant effect on the rate of spikelet initiation and resulting in an avoidance of a trade-off resulting in parallel reductions in number of spikelets.

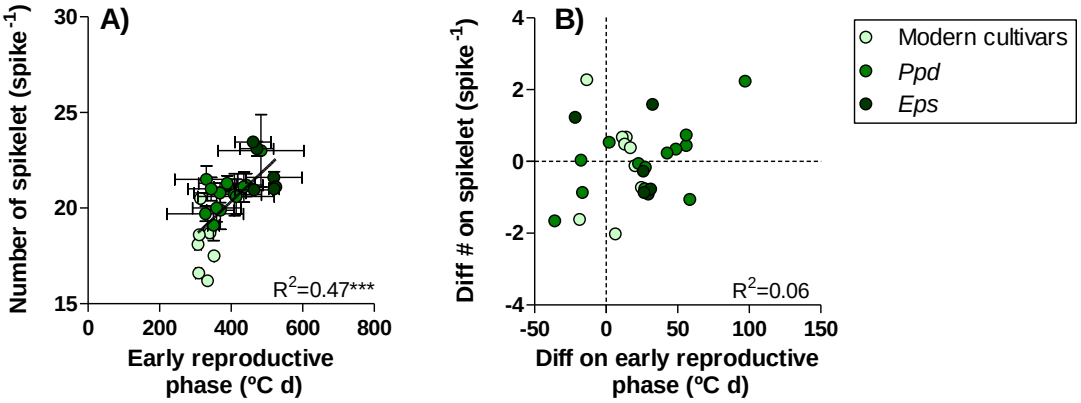


Fig.8.2. Relationship between time to anthesis and early reproductive phase (A, B) and considering the variables in each experiment in absolute values (A) or as residuals of the average of all treatments within each experiment (B). The coefficient of determination (R^2) and the level of significance (****P<0.001* and any symbols- non significant) were included.

Regarding the effects on differences in anthesis time as a consequence of changes in final leaf number and phyllochron (Jamieson *et al.*, 1998), when considering the overall variations (modern cultivars and NILs with *Ppd-1a* and *Eps* alleles introgressed across the different experiments) changes in flowering time were the consequences of changes in both FLN (Fig.8.3A) and phyllochron (Fig.8.3B).

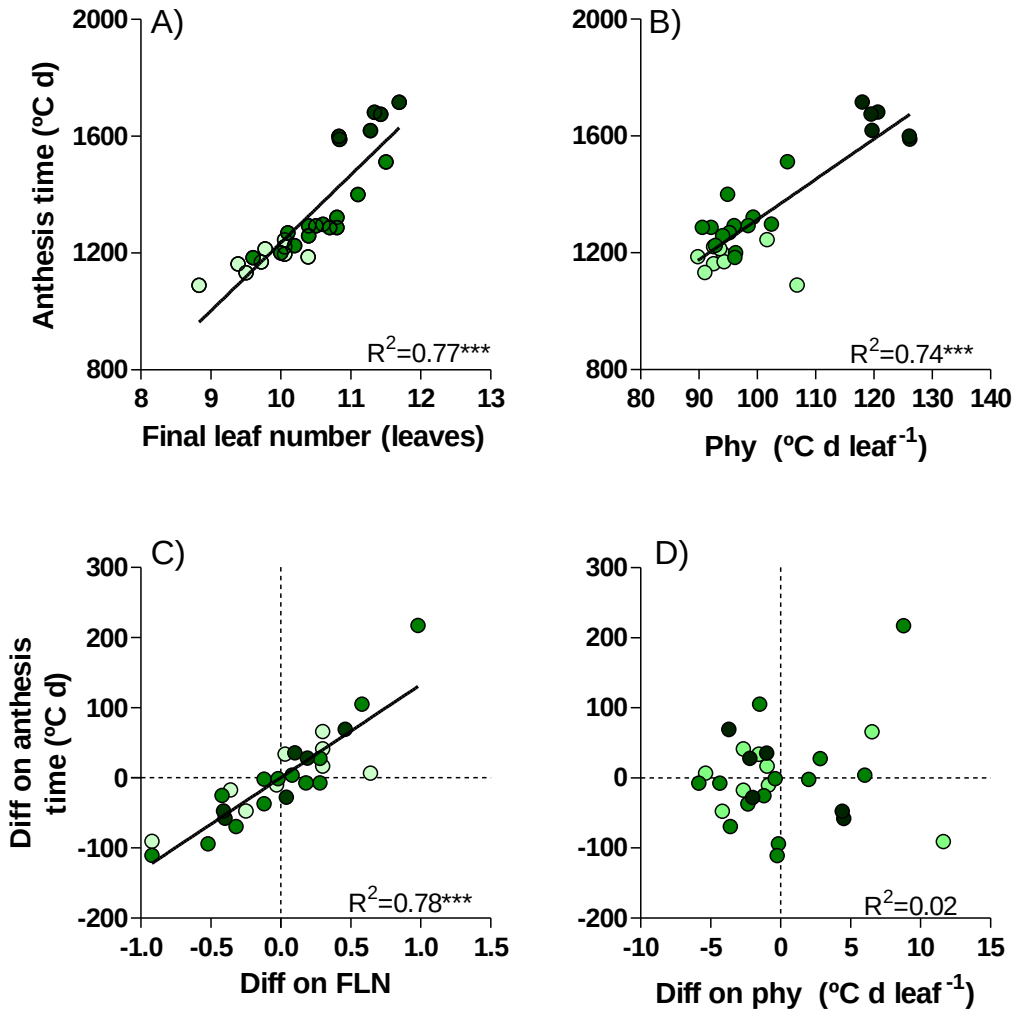


Fig.8.3. Relationship between time to anthesis and the final number of appeared leaves in the main shoot (A, C) and phyllochron (B, C) considering the variables in each experiment in absolute values (A, B) or as residuals of the average of all treatments within each experiment (C, D). The coefficient of determination (R^2) and the level of significance (****P<0.001* and any symbols- non significant) were included.

Consequently, when the whole dataset across experiments was analysed, the range of variability of FLN and phyllochron was proportionally lower than that of anthesis time (1.3 *vs* 1.6 in relative values) (Fig. 8.3A, B). But, alike when the analysis was done with the component sub-phases, a major driving force for the relationships was the different experiments rather than the differences between genotypes within experiments. Indeed both FLN was highest and phyllochron longest in the experiments with the *Eps* NILs, all the opposite in the experiments comparing modern cultivars, and intermediate in the experiments with the *Ppd* NILs (Fig.8.3A, B).

When disregarding the experiment effects to focus on the genotypic effects beyond the experimental condition in which these genotypic effects were assessed, considering the residuals of the genotypic mean for each group of genotypes within experiments, it can be seen that differences in phyllochron were mostly negligible (Fig. 8.3D) and those in FLN were critical (Fig. 8.3C) for establishing the effects of *Eps* and *Ppd* alleles or the differences among modern cultivars in time to anthesis. Although phyllochron was not a relevant trait to fine-tune flowering time in most cases analysed in this study, it might be used to achieve greater early vigour with no effects on adaptation when considering the modern genotypes or most effects of *Ppd-1* alleles, as the variation imposed by these sources of variation in FLN (and therefore in adaptation) were independent of those imposed in phyllochron (Fig 8.4), even though there were differences in the latter. Early vigour depended essentially of phyllochron of early leaves as a shorter phyllochron of early leaves might allow a rapid light interception and consequently an improved evaporation/transpiration ratio in the early stages of the crop. Then, early vigour may be associated to a faster ground cover and to soil evaporative savings during early crop development (Mullan and Reynolds, 2010). Many efforts have been carried out by breeding programs in order to profit on the genetic variation in early vigour, but the progress has been scarce. However, Richards and Lukacs, 2002 provided a large genetic variation for early vigour and this Thesis showed great opportunities of genetic variation of early vigour generated by introgression of *Ppd* alleles or likely from crosses of the elite material tested. Improving early vigour has been a target (between others) pursued by wheat breeders (Richards and Lukacs, 2002). Greater early vigour seemed to be related with a high water use efficiency (Siddique *et al.*, 1990) and improved light interception causing a higher biomass and grain yield in some environments (Rebetzke and Richards, 1999).

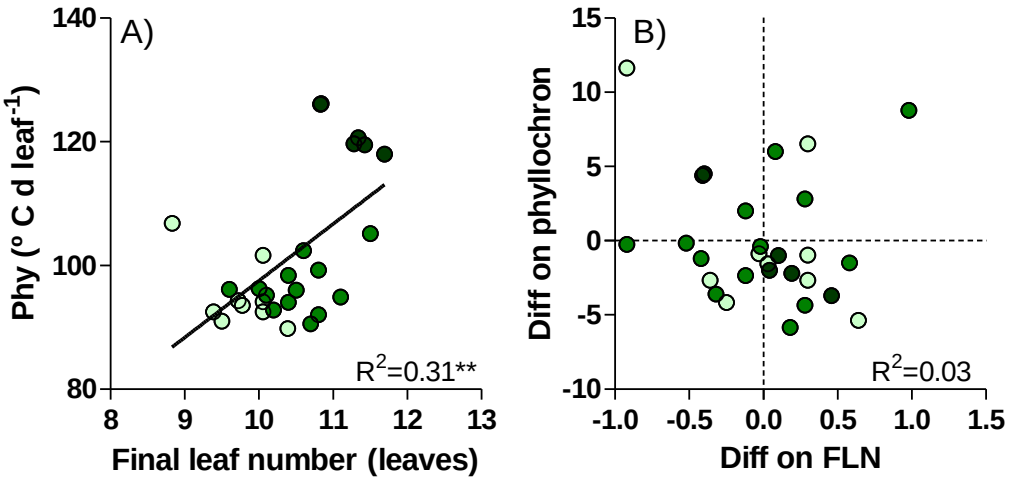


Fig.8.4. Relationship between phyllochron and the number of appeared leaves in the main shoot (Haun stage) considering the variables in each experiment in absolute values (A) or as residuals of the average of all treatments within each experiment (B). The coefficient of determination (R^2) and the level of significance (***P<0.01* and any symbols- non significant) were included.

In Table 8.1 a semi-qualitative summary of the magnitude of the effects of *Ppd* and *Eps* alleles and the genetic variation among modern genotypes is offered for the main traits studied in this Thesis. Basically, *Ppd* insensitive alleles reduced all the traits studied while the *Eps* NILs depended on the chromosome in which they were introgresssed and the donors of the alleles (Table 8.1). Regarding the nine modern wheat genotypes tested in the Thesis the main variation in flowering time was due to variations in the late reproductive phase (Table 8.1).

Table 8.1. Effects of *Ppd* and *Eps* alleles and genetic variation of modern genotypes on anthesis time, on the components of anthesis time (developmental phases, final leaf number(FLN) and phyllochron) and on number of spikelets and fertile tillers.

Genotypes		Anthesis time (°C d)	Vegetative phase (°C d)	Early reproductive phase (°C d)	Late reproductive phase (°C d)	FLN (emerged leaves)	Phyllochron (°C d leaf^{-1})	Number of spikelet (spike^{-1})	Fertile tillers (plant^{-1})
Ppd NILs	singles	++	+	=	+	+	+ (late leaves)	+	=
	doubles	++	++	+	+	++	+(late leaves)	+	=
	triples	++	+	+	++	++	+(late leaves)	+	=
Eps NILs	AXC 1D	++	=	+	+	=	=	++	=
	SxR 1D	+	+	=	+	+	=	=	=
	AxC 3A	=	=	=	=	=	=	=	=
Modern genotypes		++	+	=	++	++	+	++	+

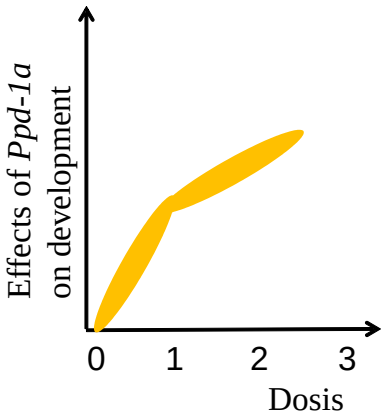
The symbols indicate no changes (=), small variation (+) and high variation (++) with respect to the Paragon or *Eps* late and among modern genotypes.
For *Ppd* alleles, it is distinguished according the number of doses of *Ppd-1a* alleles introgressed.
For *Eps* alleles, it is differentiated the effects of different donors and of location of the QTL on the traits.
For modern genotypes, it is considered the variation among the "pool" of nine genotypes.

8.3. Testing the hypothesis

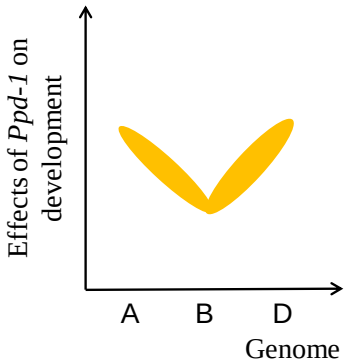
Through this Thesis there have been hypotheses, either explicit or implicit, of two different natures: some of them were related to the materials used, the others were related to the traits analysed. This is because the work developed through this thesis had novel and relevant components regarding both elements.

(*i*) Considering the materials used, and regarding the overall effects across developmental traits, the hypotheses included that

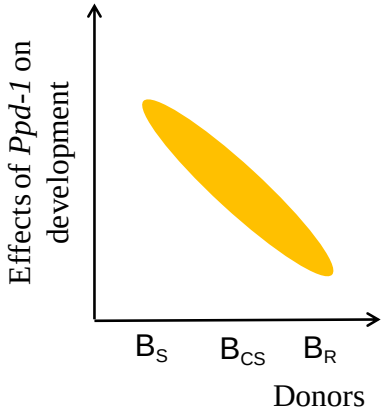
***i*.1. The effects of *Ppd-1a* alleles would be stronger with the increase in the doses of alleles introgressed. This hypothesis was accepted** as in general there was a strong effect when introgressing a single allele of insensitivity and the effects were stronger when comparing lines with more than one allele with those possessing only one. However, unlike it was expected, the effect was not additive and double/triple doses of insensitivity did not result in an acceleration of development 2-/3-fold of that produced by the introgression of a single dosis into the wild type with sensitivity alleles in all three genomes.



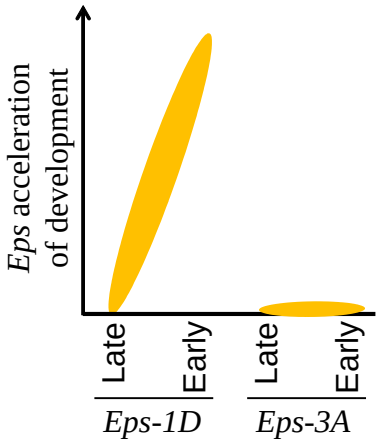
***i*.2. The effects of *Ppd-1a* alleles would depend on the genome in which these alleles were introgressed. This hypothesis was accepted.** In the literature, photoperiod insensitivity alleles located on D genome seemed to have stronger effect on development than alleles in the other two genomes, which agrees with the overall results found in this Thesis. However, it is also commonly found in the literature that the effect of *Ppd-B1a* is stronger than the allele of the A genome, and in this work we did not confirm this as *Ppd-1a* in both the A and the D genome exhibited stronger effects than when introgressed in the B genome.



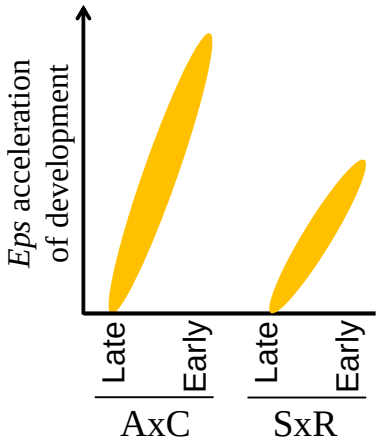
***i.* 3. The particular source of *Ppd-1a* alleles (in this Thesis exemplified with *Ppd-B1a* alleles from different donors) may be relevant when determining the magnitude of the allele effect. This hypothesis was accepted.** The effects of *Ppd-B1* alleles on developmental traits depended on donors of these alleles. Normally, Recital had the weakest effect followed by Chinese Spring and finally by Sonora 64. This may well be –at least in part- the origin of the controversies regarding the actual magnitude of the effect of *Ppd-1a* alleles depending on whether they are introgresssed in the A, B or D genome.



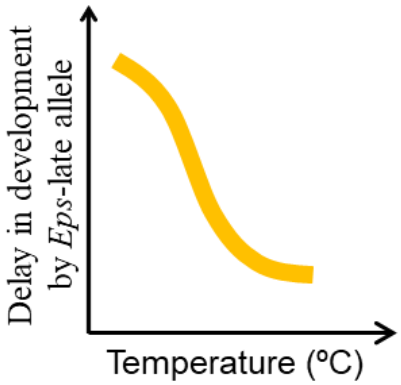
***i*.4. The *Eps* genes would affect development with differences on the magnitude depending on the location of the loci on the chromosome. This hypothesis was partially accepted.** It was accepted in that *Eps*–early alleles on chromosome 1D generated an effect that was different to that of *Eps*–early alleles on chromosome 3A; but the latter actually did not modify development at all and the hypothesis assumed that both *Eps* genes would have affected development (as in previous studies when these lines were developed). This conflict with the literature using the same *Eps*–3A, reinforced one of the original hypothesis: that the background environmental conditions (in this Thesis the growing temperature) may affect the developmental responses to the introgression of *Eps* genes; implying that despite their formal names these genes may not be "intrinsic" or "*per se*" but responding to an environmental factor (alike other, *Ppd* and *Vrn*, developmental genes), in this case, the temperature.



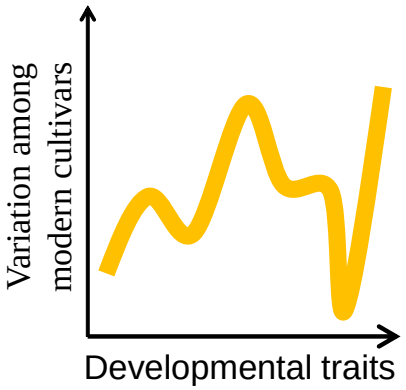
***i*.5. The magnitude of the effect of a particular *Eps*–early allele would depend on the source of the allele or on the genetic background in which the allele is introgressed. This hypothesis was accepted.** *Eps1D*–early allele from Avalon x Cadenza cross generated stronger effects than the "same" allele from Spark x Rialto cross.



***i*.6. The effects of *Eps* genes on developmental processes would be affected by the backgrounds temperature of growth. This hypothesis was accepted.** The difference between *Eps1D*–late and –early alleles was affected by temperature both in calendar and in thermal times.



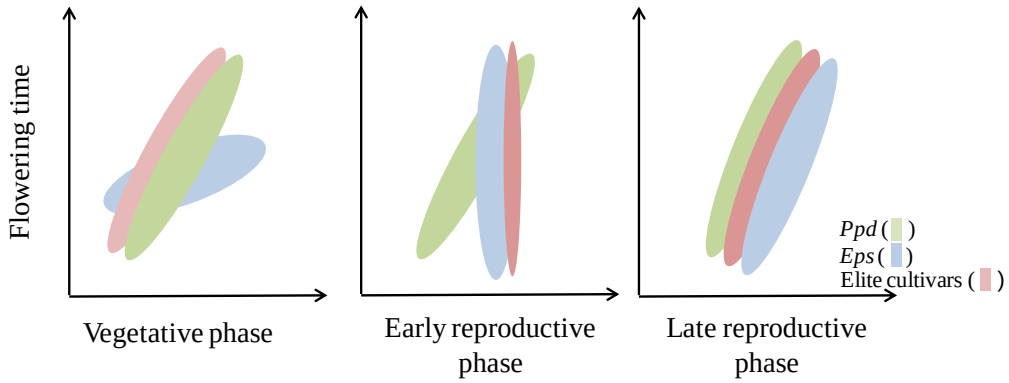
***i*.7. Even though all modern cultivars are well adapted, there would be variation available in most developmental traits. This hypothesis was accepted.** Expectedly, the final outcome of all developmental processes (namely flowering time) was relatively similar among modern and well adapted cultivars, but for many determinants of this trait, there was a variation available (of different magnitude depending on the traits) that might be amenable for breeding when selecting parents for designing strategic crosses.



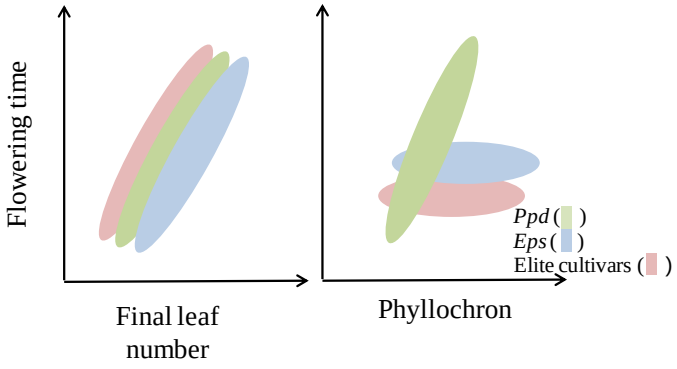
(*ii*) Taking into account the physiological traits as affected by the genetic variation generated by introgression of *Ppd* and *Eps* alleles (considering the most important effects and disregarding the effects of particular alleles) and by comparing a set of modern cultivars, the hypotheses included that the overall developmental effects reflected in time to anthesis:

***ii*.1. would be due to effects on duration of all its component phases** (vegetative, early and late reproductive phases). **This hypothesis was only partially accepted**

as it was the case for the effects of introgressing *Ppd* alleles. However, for the effects of *Eps* alleles and for variation among modern cultivars, flowering time was mainly related to duration of both vegetative and late reproductive phases (for modern cultivars) or exclusively to that of the late reproductive phase (for *Eps* alleles).

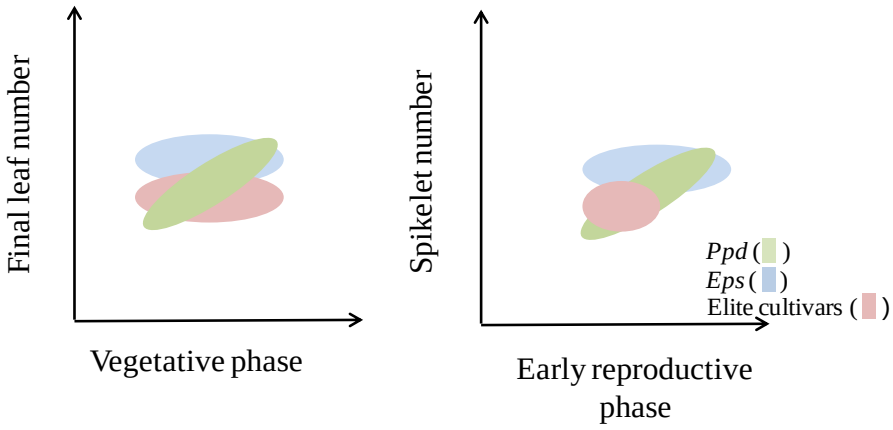


***ii*.2. would be due to effects on final leaf number and phyllochron (phyllochron of early and late leaves). This hypothesis was only partially accepted.** When changes in flowering time were conferred by introgressing *Ppd-1a* alleles the hypothesis was true as the shortening of time to flowering was caused by reductions in both FLN and phyllochron (in particular that of the late leaves). However, whilst when variation in flowering time was due to the fine-tuning produced by the introgression of *Eps* alleles or by differences among modern cultivars, it was mainly related to reduction of FLN.

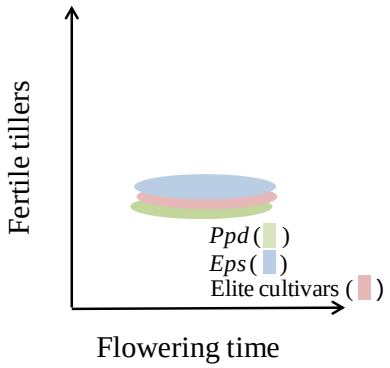


***ii*.3. through affecting the duration of particular developmental phases would produce a parallel change in the number of organs initiated in these phases (i.e. FLN and number of spikelets would be related to the length of the vegetative and early reproductive phases, respectively). This hypothesis was partially accepted**

when considering the *Ppd-1* genes and completely rejected when considering the other sets of genotypes. When *Ppd-1a* alleles accelerated development of the phases reducing their duration they also accelerated the rates of primordia initiation but less remarkably, and consequently the hypothesis was partially accepted as it was true qualitatively but quantitatively the effect was less than proportional. In none of the other cases analysed was there a clear direct relationship between the duration of the vegetative phase and the FLN. Thus, the introgressi *Eps*–early alleles produced an increase in the rate of leaf-primordia initiation similar to that produced on the rate of phasic development, shortening leaf-plastochron in parallel with the shortening of the vegetative phase; and the same occurred with the subtle variation in vegetative phase among the modern cultivars. Regarding the final number of spikelets initiated during the early reproductive phase, it was largely unaffected by *Eps* genes, or by the variation produced when comparing modern cultivars. However, as I mentioned before, when introgressing *Ppd-1a* alleles the rate of primordia initiation was increased less than the rate of phasic development for the ERP and therefore in this case there was a decrease on spikelet number when the ERP was shortened, but even in this case the hypothesis would not be fully accepted as the decrease in spikelet number was less than proportional.

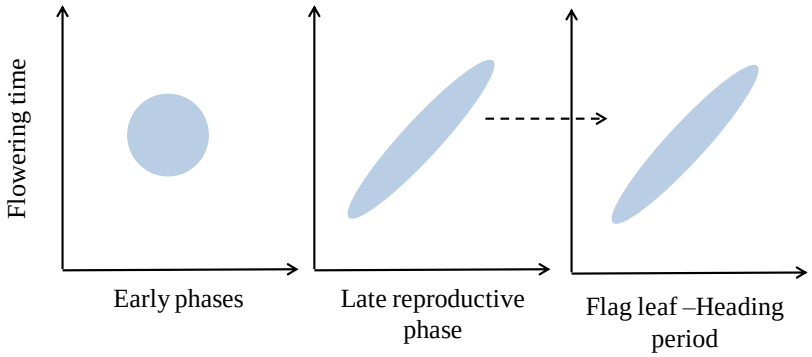


***ii*.4. would result in parallel changes in number of tillers through affecting tillering dynamics and the final outcome of them: the number of fertile (spike-bearing) tillers. This hypothesis was rejected**. Both the effects of



Ppd-1 or *Eps* genes and the variation among modern cultivars which in all cases affected time to anthesis had no clear effects on the dynamics of tillering and the small effects they might have had were compensated (i.e. reductions in maximum number of tillers was followed up by reductions in tiller mortality); consequently genotypic differences in time to anthesis did not affect the number of spikes.

***ii*.5. when affected by the *Eps* x Temperature interaction would be related to changes on the duration of all developmental phases. This hypothesis was rejected.** Reduction of flowering time by the interaction *Eps* x temperature was mainly explained by differences in the length of the late reproductive phase with no changes in the duration of early phases (beyond the universal effect of temperature) and in fact not the whole LRP but mainly the duration of the period from flag leaf emergence to flowering.



As development before TS was not affected by the interaction *Eps x temperature*, spikelet number was unaffected.

8.4. Main contributions of knowledge

Through testing the hypotheses with the experiments carried out along the research of this Thesis I obtained a large number of results. In this last section I did illustrate with a number of examples of these contributions to knowledge produced in the process (not pretending to produce a full list). In this illustration of the contributions made I divided them into the three groups of genotypic effects analysed and within them categorised the contributions from **completely novel**, through **consolidating previous** but scarce **results**, to **confirmations of knowledge** already well **established**. The completely novel category include pieces of knowledge that, as far as I am aware, have never been

produced before, the consolidating category refers to new pieces of knowledge that result relevant to strengthen previous results from what may be considered emerging knowledge, whilst the last category (the least interesting one) are contributions to knowledge simply confirming what is already well known.

About *Ppd* alleles

Confirming well established knowledge: Photoperiod insensitivity genes (i) consistently accelerates development of wheat under field conditions, (ii) with effects that are different depending on whether the genes considered are in the A, B or D genomes, and (iii) in general increasing the magnitude of the effects with increasing the doses.

Consolidating knowledge: It was found that phyllochron was better described by a bi-linear than by a linear model, with later leaves appearing slower than early leaves. Although there were some published papers demonstrating this, the vast majority of the literature simply considers phyllochron as a single value for all leaves. Furthermore, I also found that *Ppd-1a* alleles reduced phyllochron, a fact not well documented with only incipient results available in the literature. There was a trade-off between acceleration of the ERP and that of the spikelet initiation rate resulting in an effect of *Ppd-1a* alleles on the duration of the phase much larger than the effect on the number of spikelets per spike, again an effect only emergently acknowledged. It was also confirmatory that the effect of the doses of insensitivity was not additive, again a fact only fragmentarily reported so far.

Completely novel contributions: To the best of my knowledge, this is the first study that compares simultaneously NILs with different doses and sources of *Ppd-1a* alleles and the genomes in which these alleles were introgressed on a number of different developmental attributes (some only seldom determined even with less complex arrangements of NILs, like plastochron). It was found, that the source of a particular allele may be very relevant in determining the effect of the introgression in a particular genome. This novel discovery would allow elucidating the causes of conflicts so far only recognised, but not explained, in the literature regarding the relevance of particular genomes in conferring photoperiod insensitivity. Also it was shown for the first time with evidences what had been only speculated in the past regarding the possibility of tailoring the pattern of pre-anthesis development to reach a the same time to anthesis through combining particular *Ppd-1a* alleles, a knowledge that may be potentially used

to manipulate the partitioning to flowering time and likely to improve yield potential.

About *Eps* alleles

Confirming established knowledge: *Eps* alleles caused a rather small variation in flowering time, useful for fine-tuning adaption, and the magnitude of the effect actually depended on the particular *Eps* gene considered (there are a large number of them).

Consolidating knowledge: It has been reported a possible *Eps* x background interaction, and we confirmed this is likely when comparing the magnitude of the effects of *Eps1D* from two different crosses, Avalon x Cadenza and Spark x Rialto. Furthermore, as many different developmental processes were analysed, we did go a step forward to this and did not only show this likely interaction in terms of the magnitude of the effect in time to anthesis but also that the mechanisms by which the *Eps1D* gene ended up affecting time to anthesis were different. It was also confirmed that the effects of these alleles on phenology were compensated with those on spikelet initiation and, therefore, *Eps* did not significantly affect spikelet number. It was also evidenced that even though these genes are called "earliness *per se*", assuming their effect on the rate of development would be "intrinsic" and independent of the environment, they interacted with temperature, an interaction only shown before for an unusually strong *Eps* gene in diploid wheat. This interaction might be behind the fact that we did not find any effects of *Eps3A* alleles in our Mediterranean region when it was reported to affect anthesis time in the UK.

New contributions: *Eps1D*–early alleles accelerated flowering time through shortening the late reproductive phase, which is completely novel. There have been limited research on particular *Eps* genes, most of which focused on identifying them, and therefore there have been almost no works on the effects on particular phases. In this cases and owing to the general empirical fact that normally the duration of the vegetative phases are more plastic, it has been supposed that *Eps* genes affected mainly the duration of early phases of the crop. Furthermore, this Thesis is the first study showing *Eps* x temperature interaction on hexaploid wheat with only subtle *Eps* effect on heading time, and proving that the interaction was independent of the temperature effect of the difference (i.e. it was maintained when calculated in thermal time). In this context, it was shown that the nature of the interaction involved that *Eps* modified the

cardinal temperatures, something that was never hypothesised before.

About modern cultivars

Confirming established knowledge: There is only limited variation in flowering time among modern cultivars, as it is expected because cultivars must be adapted to the region and flowering time is critical for adaptation.

Consolidating knowledge: The small variation in flowering time was associated with that on the duration of late reproductive phase, rather than on the more plastic vegetative phase. Furthermore, the durations in the phases were independent of each other which would allow the manipulation within elite materials of duration of pre-flowering phases without modifying the whole period of heading time.

New contributions: there is genotypic variation in spikelet-plastochron which allows speculating with the hypothesis that it could be combined through strategic crosses particular durations of the early reproductive phase with particular durations of spikelet-plastochron increasing the likelihood of contributing to improved spike fertility.

8.5 References

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