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VARIABILITY ASSOCIATED TO THE INSERTION AND EXPRESSION OF TRANSGENES IN PLANTS

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Dra. Maria Pla i de Solà-Morales, Lecturer in the Food Technology Area, in the Chemical and Agricultural Engineering and Food Technology Department, and Researcher in the Institute of Food and Technology at the University of Girona,

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CERTIFY:

That they have supervised the work carried out by Anna Coll Rius in the Food Technology Area at the University of Girona entitled *Variability associated to the insertion and expression of transgenes in plants*, which is submitted as a paper-compendium format in this dissertation to apply for the Doctor degree by the University of Girona. All the requirements to be submitted as a paper-compendium format and to get the European mention are complied.

Three papers are included in this dissertation, and Anna Coll Rius is the first author in all three papers. Two of them were published in Plant Molecular Biology in 2008 and 2010, a journal in the first quartile of Plant Science category in 2008 (the impact factors for 2009 and 2010 are not still available). Plant Molecular Biology was in the 17th position of 153 in 2008, with an impact factor of 3.541. Another paper was published in Transgenic Research in 2009. This journal was in the second quartile of Biotechnology and Applied Microbiology category in 2008 (the impact factor for 2009 is not still available): it was in the 41st position of 143 and had an impact factor of 2.809. The dissertation includes as well a manuscript submitted for publication.

All studies were focused on gene expression and protein variability between transgenic and non-genetically modified comparable plants, using as model the maize commercial event MON810, the only GMO cultured in the EU and widely planted in Catalunya.

Dra. Maria Pla i de Solà-Morales

Dra. Anna Nadal i Matamala

Girona, March 2010

Aquest treball s'ha pogut dur a terme gràcies al suport del Fons Social Europeu i del Departament d'Universitats, Recerca i Societat de la Informació, mitjançant una beca predoctoral FI de l'Agència de Gestions i Ajuts Universitaris i de Recerca, de la Generalitat de Catalunya.

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Coll A, Nadal A, Collado R, Capellades G, Messeguer J, Melé E, Palaudelmàs M, and Pla M (2009). Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in the field are more similar than are those of conventional lines. *Transgenic Res.* 18, 801-808.

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SUMMARY

To ensure the safety of consumers and the environment, genetically modified (GM) food and feed are submitted to strict legislation in many countries. The EU legislation establishes an authorisation procedure requiring: molecular characterisation of the transgene, compositional comparative analysis, food/feed safety assessment, environmental risk assessment and post-marketing environmental monitoring plan. The use of profiling approaches to evaluate the possible occurrence of unintended effects derived from the insertion and/or expression of the transgene has been proposed as an interesting complementary tool for safety assessment.

The objective of this thesis was to evaluate the variability associated to the insertion and expression of transgenes in plants, using as example MON810 maize i.e. the only GMO event that is authorised for commercialisation and cultivation in the EU. We aim to complement the existing targeted approaches by providing more unbiased information on the basis of profiling techniques.

We initially used microarray hybridisation to compare the transcriptomic profiles of two MON810 and non-GM near-isogenic variety pairs. To avoid the effect of factors unrelated to the transgenic character, in vitro cultured plantlets were analysed. Analysis of ~1/3rd maize transcripts revealed transcriptomic differences of 1.7 and 0.1% in Aristis Bt/Aristis and PR33P67/PR33P66, respectively. In contrast, maize varieties obtained by conventional breeding differed in 4% analysed transcripts. No sequence could be identified that was consistently regulated in five commercial variety pairs analysed. Consequently, just a reduced number of genes were differentially expressed in MON810 and non-GM varieties; and their varietal dependence (in terms of both, numbers and identities) pointed towards the lack of direct effects of MON810 transgene.

Due to the agricultural importance of this crop and to its high use in Catalunya (where above 50% planted maize is MON810), we evaluated if these differences were kept in plants of the same varieties, at the identical development stage and grown in real agricultural fields according to conventional farming practices in the region. Reverse transcription-real-time PCR (RT-qPCR) based analysis of around 10% sequences regulated in plantlets grown in vitro showed they had similar expression levels in MON810 and non-GM varieties cultured in natural conditions. This further suggested MON810 and near-isogenic varieties are substantially equivalent, although we could not rule out the existence of other transcriptomic differences.

We then used microarray technology to evaluate unintended effects of MON810 transgene in maize varieties grown under real field conditions, taking into consideration the variability among diverse varieties obtained by conventional breeding and the effects of different farming practices. Helen Bt and Helen leaves differed in less than 0.13% transcripts both under conventional and low-N fertilisation conditions; and most differentially expressed sequences were regulated in a variety specific manner. Chronic low-N stress in agricultural fields resulted in less than 0.17% differentially expressed sequences in maize leaves.

However, they were mostly equally regulated in MON810 and non-GM varieties. Taken together, our results showed that natural variability among conventional varieties and farming practices explained most gene expression variability between MON810 and non-GM plants grown in agronomic fields. The transgenic character only explained 9.7% of the observed variability.

We finally complemented the transcriptomics approach with a proteomic analysis of maize in an economically relevant scenario. MON810 and comparable non-GM grains were grown in agricultural fields and analysed at the development stage typically used for forage (~75% maize is used for forage in the EU). Proteomic patterns of two variety pairs (PR33P67/PR33P66 and DKC6575/Tietar, the most representative in the two FAO maturity groups grown in the region) had less than 1.19% differentially accumulated proteins (at accumulation ratios below 1.8-fold), and they were not the same in different variety pairs.

In conclusion, from the transcriptomics and proteomics perspectives, MON810 maize varieties seem to be substantially equivalent to their non-GM comparators. Thus, the production of GM plants with minimal unexpected effects is possible.

RESUM

En molts països i amb la finalitat de garantir la seguretat dels consumidors i del medi ambient, els aliments modificats genèticament (MG) destinats, tant a humans com animals, estan sotmesos a una rigorosa legislació. El marc regulatori de la Unió Europea (UE) estableix un procés d'autorització pel qual els aliments MG han de superar diverses avaluacions que inclouen: caracterització molecular del transgèn, anàlisis comparatives a nivell nutricional, avaluació de la seguretat alimentària, avaluació del risc ambiental i plans de monitorització ambiental en la fase de post-comercialització. L'ús de tècniques de *profiling* per avaluar la possible existència d'efectes no esperats derivats de la inserció i/o expressió del transgèn s'ha proposat com a una eina molt interessant i alhora complementària per l'avaluació de la seguretat alimentària.

L'objectiu de la present tesis és avaluar la variabilitat associada a la inserció i expressió de transgens en plantes, utilitzant com a exemple el blat de moro MON810; és a dir, l'únic event MG autoritzat per a comercialització i cultiu a la UE. Es pretén complementar les aproximacions ja existents basades en l'estudi de paràmetres concrets aportant informació, no esbiaixada, mitjançant les tècniques de *profiling*.

Inicialment s'utilitzaren *microarrays* amb l'objectiu de comparar els perfils transcriptòmics de dues parelles (MON810 i no-MG) de varietats. Per tal d'evitar els efectes de factors no relacionats amb el caràcter transgènic es van analitzar plàntules crescudes in vitro, és a dir en cambres de cultiu en condicions molt controlades. L'anàlisi de ~ 1/3 dels transcrits de blat de moro va mostrar diferències transcriptòmiques corresponents a 1.7 i 0.1% en les parelles de varietats Aristis Bt/Aristis i PR33P67/PR33P66, respectivament. Per contra, la comparació dels perfils transcriptòmics de les varietats de blat de moro obtingudes per millora convencional va indicar que un 4% dels trànscrits analitzats tenien un patró d'expressió diferent. A més, no es va identificar cap seqüència que fos regulada consistentment en cinc parelles de varietats comercials analitzades. Així doncs, únicament un reduït nombre de gens s'expressen diferencialment en varietats MON810 i les seves corresponents no-MG; i aquesta dependència varietal suggereix l'absència d'efectes directes del transgèn MON810.

Degut a la importància d'aquest cultiu en l'agricultura i al seu elevat ús a Catalunya (on més del 50% del blat de moro sembrat és MON810), s'avaluà si les diferències observades es mantenien en condicions de camp. Per a respondre la pregunta es van utilitzar les mateixes varietats de blat de moro, a idèntic estat de desenvolupament (estadi vegetatiu de 2 fulles) i sembrades en camps reals d'agricultor seguint les pràctiques agrícoles de la zona. L'anàlisi, mitjançant transcripció reversa seguida de PCR a temps real (RT-qPCR), d'aproximadament un 10% de les seqüències regulades en plàntules crescudes in vitro, mostrà que aquestes presentaven nivells d'expressió similars en varietats MON810 i no-MG cultivades en condicions de camp. Aquests resultats suggereixen de nou l'equivalència substancial de les varietats MON810 i les seves corresponents convencionals. Tot i això, no es poden descartar l'existència d'altres diferències transcriptòmiques.

Seguidament es va utilitzar la tecnologia dels *microarrays* per avaluar els efectes no esperats del transgèn MON810 en varietats de blat de moro crescudes en condicions reals de camp. Es van incloure a l'assaig dues parelles de varietats per tal de tenir en compte la variabilitat present entre varietats obtingudes per millora convencional i dos tractaments de fertilització amb la finalitat d'avaluar els efectes de diferents pràctiques agrícoles. Les fulles de Helen Bt i Helen presentaren diferències en menys del 13% dels trànscrits, independentment del tractament de fertilització amb nitrogen (N) aplicat (control o N-reduït); i la majoria de les seqüències expressades diferencialment eren regulades de manera específica de varietat. L'estrès suau i crònic de N en camps d'agricultor va ocasionar l'expressió diferencial de menys del 0.17% dels trànscrits. Tot i això, en aquest cas la majoria de seqüències estaven igualment regulades tant en les varietats MON810 com en les no-MG. Prenent conjuntament tots els resultats obtinguts en el present estudi, s'observà que la variabilitat natural entre varietats convencionals i les pràctiques agrícoles explicava la major part de la variabilitat tanscripcional obtinguda entre el blat de moro MON810 i el no-MG cultivat en camp.

Finalment l'aproximació transcriptòmica va ésser complementada amb l'anàlisi proteòmica de blat de moro. L'anàlisi es va aplicar en mostres reals de camp i econòmicament rellevants. Es van utilitzar grans de blat de moro MON810 i no-MG en l'estadi de desenvolupament típicament destinat a farratge (a la UE ~75% del blat de moro és utilitzat per a farratge). Els patrons proteòmics de dues parelles de varietats (PR33P67/PR33P66 i DKC6575/Tietar, les més representatives dels dos grups de maduració segons la FAO cultivats en la regió) presentaren menys d'un 1.19% de proteïnes amb acumulació diferencial (amb proporcions d'acumulació per sota d'1.8 vegades) i aquestes no eren les mateixes en els diferents parells de varietats.

Per concloure, des del punt de vista transcriptòmic i proteòmic, les varietats de blat de moro MON810 semblen ser substancialment equivalents a les seves varietats comparables no-MG. En conseqüència, és possible la producció de plantes MG amb el mínim d'efectes no esperats.

ABREVIATIONS

2DE	Two-dimensional gel electrophoresis
amiRNA	Artificial microRNA
CaMV	Cauliflower mosaic virus
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
CHO	Carbohydrate
CRL	Community Reference Laboratory
cRNA	Anti-sense copy RNA
DNA	Deoxyribonucleic acid
e35S-P	Enhanced 35S promoter
EC	European Commission
EFSA	European Food Safety Authority
ENGL	European Network of GMO Laboratories
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ESI	Electrospray ionisation
EU	European Union
FAO	Food and Agricultural Organisation
gfp	Green fluorescent protein gene
GM	Genetically modified
GMO	Genetically modified organisms
GUS	b-glucoronisase
hpt	Hygromycin phosphotransferase gene
hsp	Heat-sock protein gene
IEF	Isoelectric focusing
ILSI	International Life Sciences Institute
ISAAA	International Service for the Acquisition of Agri-biotech Applications
IT	Ion trap
JRC	Joint Research Centre
LC	Liquid chromatography
MALDI	Matrix assisted laser desortion ionisation
mRNA	Messenger RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Mw	Molecular weight
N	Nitrogen
NOS-T	Nopaline synthase 3' terminator
nptII	Neomycin phosphotransferase gene
OECD	Organisation for Economic Co-ordination and Development
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
pI	Isoelectric point
Q	Quadripole
qPCR	Real-time PCR
RMA	Robust multi array average
RNA	Rybonucleic acid
RT-qPCR	Reverse transcription qPCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T-DNA	Transfer DNA
Ti plasmid	Tumor-inducing plasmid
TOF	Time of flight
T-strand	Transfer strand
V2	Vegetative two-leaf stage
vir	Virulence gene
VT	Vegetative tasseling stage
WHO	United Nations World Health Organisation

TABLES AND FIGURES

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Genetically modified (GM) plants are plants that have been modified by genetic engineering techniques and their development constitute a great technological innovation with a wide range of applications. In the field of plant breeding, transgenic plants offer interesting opportunities which is illustrated by the increasing growth rate of global biotech area and the quick incorporation of crops with new properties. Although a strict regulation governs the authorisation and use of genetically modified organisms (GMOs), one of the main consumers' concerns is the incidence of possible unintended alterations in their composition. This critical point will be in deep set out throughout this work.

1. Genetic transformation

GMOs are defined, according to the European Union (EU) (Directive 2001/18/EC), as organisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Therefore, only plants obtained by 'modern technology' are considered as transgenic plants and this technology includes recombinant deoxyribonucleic acid (DNA) techniques, methods for direct introduction of DNA into cells and cell or protoplast fusion techniques (where the fusion takes place by means of methods that do not occur naturally). In vitro fertilisation, natural transformation and polyploidy induction are specifically excluded from the definition. Moreover, mutagenesis and cell (or protoplast) fusion of organisms which can exchange genetic material through traditional breeding methods do not result in GMOs.

The current definition of GMO (based on the application of genetic modification techniques) gave rise to a controversy involving cisgenic plants. Actually, cisgenc plants are GM plants in which altered genes (cisgenes), including all additional sequences such as introns, promoters and terminators are isolated from a crossable donor plant (i.e. it could be used in traditional plant breeding) (Schouten et al., 2006b). On the other hand, transgenic plants are GM plants that contain one or more genes from non-crossable specie(s) (transgene), which represent a completely new gene pool to breed this plant species (www.cisgenesis.com/index.php). Although cisgenesis and transgenesis both use the same genetic modification technologies a number of researchers (Schouten et al., 2006a) defend that they are fundamentally different. Due to its higher relevance in terms of food safety (there is lack of agreement on

how to treat cisgenesis from the food safety perspective) (Kok et al., 2008) this thesis focuses on transgenic GM plants.

Although GM plants are defined according to the technique used to introduce foreign genes into a vegetal cell, this is not the only process involved in the genetic modification of plants. In fact, plant transformation technology used to obtain transgenic plants comprises two key steps:

- DNA insertion into the host genome
- Clonal propagation and regeneration of a transformed cell into a plant

1.1. Methods for DNA delivery

Within the 'modern technology' described in the Directive 2001/18/EC, the most widely used methods to introduce a new DNA fragment into the plant genome are: (i) *Agrobacterium tumefaciens* mediated transformation and (ii) microprojectile bombardment of intact cells or tissues.

In the first case, the Gram-negative phytopathogenic bacteria *A.tumefaciens,* capable of natural transfer of DNA into a eukaryotic host, is used as a tool for plant genetic engineering. *Agrobacterium* genetically transforms its host by transferring a well-defined DNA segment (T-DNA) from the tumor-inducing (Ti) plasmid to the host-cell genome (Gelvin, 1998). The T-DNA carries a set of genes encoding proteins implicated in the regulation of plant growth and opine catabolism enzymes. The transformation process (Figure 1) has been extensively studied and is described in several reviews (Gelvin, 2003; Lacroix et al., 2006; Tzfira and Citovsky, 2006; Vasil, 2008; Veluthambi et al., 2003). It starts when small phenolic compounds and sugars produced by wounded plant cells allow recognition and bacteria-plant attachment. This attachment induces coordinated expression of the Ti-plasmid encoded virulence (*vir*) genes which carry out essential functions for T-DNA transference (Stachel et al., 1986). The T-DNA fragment is delimited by its two borders (right and left border), 25 bp direct repeats which are transferred independently on the nature of the DNA sequence between them. Therefore, replacement of the original T-DNA genes by other genes of interest is possible and has converted *Agrobacterium* into a valuable tool for plant genetic transformation. In bacterial cells, the transfer strand (T-strand) is covalently linked to Vir proteins (T-complex) in order to be protected against cellular nucleases (Citovsky et al., 1989) and facilitate its nuclear import within the host cell. This could also take place inside the host cell cytoplasm. Once in the host cell cytoplasm the T-complex enters the cell nucleus in order to be integrated into the plant genome. Finally, the T-strand is converted into a double-strand and is integrated into the host genome by a non-homologous recombination process mediated by the cooperation between bacterial factors and components of the host cellular and nuclear machineries.

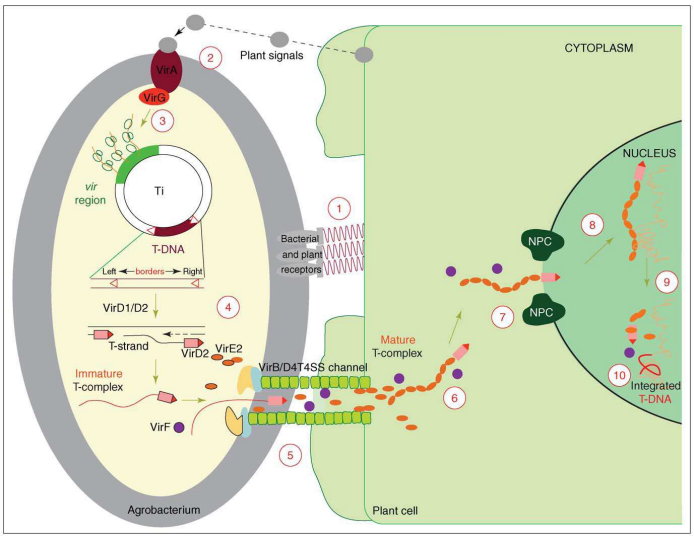

Figure 1. A model for the *Agrobacterium*-mediated genetic transformation. Transformation process is summarised in 10 steps: (1) *Agrobacterium*-plant attachment; (2) recognition of plant signals by the bacterial components *virA* and *virG*; (3) expression of the *vir* genes; (4) generation of a mobile copy of the T-DNA; (5) exportation of the immature T-DNA complex to the host-cell; (6) association of the virE2 with the T-DNA and formation of the mature T-DNA complex; (7) T-DNA complex nuclear transport; (8) recruiting of the T-DNA to the point of integration; (9) T-DNA stripped of its proteins; (10) integration of the T-DNA into the host genome. (Extracted from Tzfira and Citovsky, 2006).

From the first identification of *Agrobacterium* as the causative agent for crown gall disease of plants in 1907 (Smith and Townsend, 1907) to its application to plant genetic transformation (Barton et al., 1983; Herrera-Estrella et al., 1983) many years of research and technical difficulties have elapsed. A major advance was made by Hoekema and collegues (Hoekema et al., 1983) who modified the Ti plasmid into a binary vector system. This system includes a plasmid with the T-DNA region and a disarmed Ti plasmid without the tumour-inducing genes but retaining the *vir* loci whose products allow DNA transfer to the plant cell. Subsequent improvements in the Ti binary plasmid vectors together with modifications of transformation conditions permitted overcoming one of the major drawbacks of the technique: the transfer of DNA to monocotyledonous plants. Nowadays, this technology is considered as the first option in plant genetic transformation. The range of monocotyledonous species successfully transformed includes rice, wheat, maize, sorghum and barley (Veluthambi et al., 2003). Moreover, T-DNA has also been transferred to non plant hosts such as yeast (Piers et al., 1996), filamentous fungi (Michielse et al., 2005), cultivated mushrooms (de Groot et al., 1998) and human cells (Kunik et al., 2001).

Although the insertion of superfluous DNA (extra whole or partial copies of the transgene, vector backbone DNA or filler DNA) has been described (Afolabi et al., 2004; Forsbach et al., 2003), *Agrobacterium*-mediated gen transfer has multiple advantages in comparison to alternative methods. It can introduce long stretches of T-DNA with minimal rearrangement and high frequency of one single copy transformation events (Shewry et al., 2008).

A widely used alternative to introduce foreign genes into a plant cell is the particle bombardment technology or biolistics. It is a simple approach which involves the adsorption of DNA onto the surface of inherent metal microparticles, usually golden ones, subsequently driven at high speed onto plant cells (Sandford et al., 1993). Once there, a small portion of DNA can enter the cell nucleus and is integrated into the plant genome. There are three main systems for particle bombardment (Veluthambi et al., 2003). The Biolistic® PDS-1000/He Particle Delivery System (Kikkert, 1993), powered by a burst of helium gas that accelerates microparticles, has been the most extensively used method. But particle inflow gun (PIG) and ACCEL™ electric discharge particle acceleration devices have also been successfully used (Shewry et al., 2008).

Biolistics was first used to shoot DNA-coated tungsten particles into onion epidermal cells (Klein et al., 1987) and within a few years it was applied to transform a wide variety of plant species. Due to the inability to transfer DNA to monocotyledonous plants by *Agrobacterium* during years, it was used to produce the first transgenic plants for many economically important cereals such as maize (Fromm et al., 1990), sugarcane (Bower and Birch, 1992), wheat (Vasil et al., 1992), rice (Christou et al., 1991) and cotton (McCabe and Martinell, 1993).

Successful applications of particle bombardment indicate that it is a potent and effective transformation procedure, especially when the *Agrobacterium* based protocol does not produce satisfactory results. However, it appears to cause gene silencing, genomic rearrangements and rarely gives simple integration patterns (single copy) (Filipecki and Malepszy, 2006). In fact, the impact on the plant genome caused by different transformation techniques has been widely discussed. For instance, Labra and co-workers (Labra et al., 2001) demonstrated that rice produced by particle bombardment is characterised by far, higher genomic changes than is rice produced by *Agrobacterium*. So, although particle bombardment allows transferring DNA to a wide range of cells and tissues from a great diversity of organisms, the complex patterns of DNA integration resulting from this methodology made a number of researchers switch to a 'cleaner' technology such as *Agrobacterium*-mediated gene transfer.

1.2. Tissue culture: regeneration, selection and acclimatisation

Stable transformation protocols in general require an in vitro regeneration step in order to obtain whole plants from genetically transformed plant cells. Two main routes to recover plants have been described: organogenesis and somatic embryogenesis (Shewry et al., 2008). Organogenesis relies on the production of adventitious organs arising from meristematic tissues. On the other hand, somatic embryogenesis involves formation of embryo-like structures from somatic tissues.

Due to the low efficiencies of DNA integration, a selection system during the tissue culture phase is necessary. The objective is, in general, to avoid or compromise the growth of untransformed cells and contribute to the preferential growth of transformed cells. Selection

systems comprise two components: tissue culture media with a selection agent and a selectable marker gene incorporated into the transformation cassette which confers advantage under particular media conditions (Shewry et al., 2008).

Since the first transgenic plants, antibiotic and herbicide resistance selectable marker genes are widely used (Sundar and Sakthivel, 2008). Specifically, the most commonly applied ones are:

- The *nptII* gene: encode the enzyme neomycin phosphotransferase which inactivates aminoglycoside antibiotics (Bevan et al., 1983; Fraley et al., 1983). Kanamycin selection is the antibiotic commonly used in this selection system.
- The *hpt* gene isolated from *Escherichia coli,* codes for the enzyme hygromycin phosphotransferase and confers resistance to the herbicide hygromycin B (Waldron et al., 1985).
- The gene *bar* from *Streptomyces hygroscopicus* encodes the enzyme phosphinothricin acetyltransferase (PAT) (Waldron et al., 1985; White et al., 1990). The enzyme converts ammonium glufosianate (PPT), active principle of many commercial herbicide formulations (e.g. Bialaphos, Finale or Basta), in a non-toxic acetylated form. Consequently, only cells which have incorporated the *bar* gene will growth in the presence of PPT.

One of the major concerns in terms of human, animal and environmental safety is related to the horizontal and vertical transfer of transgenes, including selectable marker genes. The use of marker genes not based on antibiotic or herbicide resistance genes solves to some extent this problem (Darbani et al., 2007; ISAAA, 2009). New selection systems based on the use of positive selection markers which are considered more environmentally benign, have recently been developed (Shewry et al., 2008). The *manA* gene isolated from *E.coli* (Miles and Guest, 1984) is an example. This gene encodes for the enzyme phosphomannose isomerase (PMI) which converts mannose-6-phosphate to fructose-6-phospate and allows transformed cells to use mannose as a source of carbohydrate (Joersbo et al., 1998).

Optionally, reporter genes which allow making transgenic plants visually recognizable can be used. This approach does not compromise the growth of untransformed cells, thus transformed and non-transformed cells must be manually separated (ISAAA, 2009). Within this alternative, the most commonly used genes are:

- *uidA* gene, isolated from *E. coli*, encodes the β-glucoronisase (GUS) enzyme (Jefferson et al., 1986). This enzyme hydrolyses the uncoloured substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuside (X-GLU) in a compound (diXH-indigo) that forms blue precipitates. It requires externally added substrate which is destructive for the evaluated tissue.
- Green fluorescent protein *(gfp)* gene from *Aequorea victoria* encodes a protein that emits green fluorescent light when is exposed to ultraviolet light (Ormo et al., 1996). It allows monitoring plant transgene expression in real time, in live

cells or intact plants. Although GFP is widely used as a visible reporter in genetic transformation, other fluorescent proteins including AmCyan, ZsGreen, AsRed, DsRed, HcRed and ZsYellow are also available.

Additionally, research efforts have been directed towards the development of marker-free GM plants through segregation of marker genes from the host genome after regeneration of transgenic plants. Co-transformation is the simplest marker removal approach developed so far. It is based on co-integration of the marker gene and the transgene of interest into different unlinked locations in the host genome and their subsequent segregation in the progenies. Three different co-transformation methods can be carried out, based on: (i) introduction of two T-DNAs into two separate *Agrobacterium* strains, (ii) the use of a bacterial strain carrying two independent vectors and (iii) the introduction of two T-DNAs at separate sites of the same vector. In any case, the selectable marker gene can be removed from the host genome along the gene segregation process which takes place in sexual reproduction. Alternatively, selectable marker genes can be removed from the plant genome by means of excision mediated by site-specific recombination, transposons or intrachromosomal recombination.

After regeneration of GM plants from transformed cells, its transfer from in vitro conditions to a greenhouse or filed environment is a critical step. Actually, during in vitro culture plantlets grow in cultivation vessels under very special conditions: restricted air exchange, low levels of light, high level of humidity, aseptic conditions and a medium supplemented with sugar, nutrients and growth regulators. These conditions contribute to the formation of plantlets with altered morphology (apical dominance and absence of roots), anatomy (miniaturisation, silk absence and thinner cuticle) and physiology (heterotrophic growth) which can not survive environmental greenhouse or field conditions. Therefore, after ex vivo transplantation plantlets need some weeks of gradual acclimatisation. Details of acclimatisation and its success mostly depend on the species.

At this point it has to be highlighted that the appearance of the floral dip transformation protocol (Clough, 1998) markedly advanced the ease of obtaining transformants. It is an in planta method (i.e. genes are delivered into intact plants) thus avoiding the tissue culture step, saving time and skilled labor. Transforamtion of female gametes is accomplished by dipping plant inflorescences into an approtiate strain of *Agrobacterium* carrying the gen(s) to be transferred, next seeds collected from these plants are germinated under selection to identify transgenic individuals (Bent, 2006). For ten years the floral dip transformation method was only successful with *Arabidopsis thaliana* and some other Brassicaceae (Curtis, 2001; Clough, 1998; Tague, 2001; Wang, 2003b). More recently, the method has also been applied to wheat (Zale, 2009).

2. Transgenesis applications

GM plants appeared in the scientific literature in 1983, when the introduction of foreign genes into plants was demonstrated by two different research teams (Barton et al., 1983; Herrera-Estrella et al., 1983). Since then, the interest in plant genetic transformation arose in several ways as a new opened door to many applications.

2.1. Transgenic plants and research

Transgenic plants are a powerful tool in plant biology research. There is no doubt that the introduction of a new gene or its suppression opens a great range of possibilities for both, basic and applied research. Transgenic plants have allowed researchers to study plant physiology precisely, its biological development or its response to different stresses.

As an example of transgenic plants' application in research, the expression levels of a gene can be altered by genetic engineering in order to gain an understanding of the gene's function. Specifically, technologies such as knock-down or gene silencing aim at gene expression reduction. Several tools allow these strategies, for instance antisense mediated gene silencing (Bourque, 1995), cosuppression (Napoli et al., 1990; van der Krol et al., 1990), interference RNA (iRNA) (Baulcombe, 2000) and virus-induced gene silencing (VIGS) (Fire et al., 1998) which are post-trancriptional gene silencing approaches. They all converge in the synthesis of double-stranded (ds) RNA (Vance and Vaucheret, 2001) which is cleaved by a specific ribonuclease to produce double-stranded fragments of 21-24 bp called small interfering RNA (siRNA) that are then integrated into an active RNA-induced silencing complex (RISC) and guided to the homologous target messenger RNA (mRNA), knocking down the expression of the gene. Although this methodology is a powerful tool in functional genomics studies of plants, the specificity was shown to be an important drawback of transgene-mediated gene silencing (Xu et al., 2006). Recently developed artificial microRNAs (amiRNAs) use endogenous microRNA (miRNA) precursors to generate a single specific small RNA (sRNA) in vivo. Endogenous miRNA is a 21-base-long region of a non-coding RNA precursor that forms double-stranded hairpin structures. In knock-down experiments, mature miRNA region of miRNA precursor is replaced with the duplex sequence of amiRNA designed to specifically target a selected gene. This amiRNA-based gene silencing technique was demonstrated to be effective in *Arabidopsis* (Alvarez et al., 2006; Schwab et al., 2006), tobacco (Alvarez et al., 2006), tomato (Alvarez et al., 2006) and rice (Warthmann et al., 2008).

The technologies described above have been effective in studying gene function in plants but they are not useful for functional genomics and large-scale silencing programmes (Sallaud et al., 2004). Insertional mutagenesis is based on random insertion of foreign DNA into the host genome using transposable elements or T-DNA in order to generate a large population of mutants. Several reports show the application of this technology in large-scale surveys of gene function in *Arabidopsis* (Szabados et al., 2002), tomato (Gidoni et al., 2003) and rice (Sallaud et al., 2004).

In the frame of research applications, not only stable transformed plants can be used but also plants with transient expression of an introduced gene have a high value. Actually transient assays are quick, efficient, provide important information and somehow reflect the in vivo situation in planta (Lu et al., 1998). Nowadays, *Agrobacterium*-mediated transient assays and in particular, leaf agroinfiltration has become the technique of choice in a wide range of plant molecular biology studies since it is an easy and non invasive tool (Wroblewski et al., 2005). A particular case of employment of this approach is in the field of characterisation of promoters and transcription factors. Just as examples, agroinfiltration allowed to demonstrate that *TaNAC4* gene functions as a transcriptional activator involved in wheat response to biotic and abiotic stresses (Xia et al., 2010) and to characterise the crucial elements for pathogen response of a lipid transfer protein (*LTP*) promoter from pepper (Jung et al., 2005).

2.2. Transgenesis as a tool for plant breeding

There is no doubt that genetic engineering techniques have aroused a great interest in the frame of plant breeding. Genetic modification, similar to classical breeding, aims at producing plants with improved properties in terms of agronomic, nutritional and/or processing quality. However, it additionally appeared as a tool to introduce completely new traits. In fact, classical breeding methods allow obtaining improved plant varieties by crossing different lines; consequently they rely on the genetic variability of this particular species. If the desired characteristic is not present within crossable varieties (even if they are distantly related) mutant lines obtained by chemical mutagens or ionizing radiation (Ahloowalia and Maluszynski, 2001) could be an alternative (although mutations are unpredictable). But transgenesis can be, at least in some cases, the tool of choice since it allows transferring essentially only the gene of interest to a plant.

After 14 years on the market, just a few GMOs are commercially available and most of them have been modified to increase the yield and/or to facilitate crop management. Actually plants with improved farming characteristics (e.g. herbicide tolerance, insect resistance, virus resistance or delayed ripening), based on the insertion of a single gene which primarily benefits growers, were the initial focus of genetic engineering and constituted the so-called 'first generation GM plants'. Among them, herbicide tolerance has been the most widely adopted trait (James, 2009). It simplifies weed control by enabling farmers to use high-spectrum herbicides at emergence time and consequently, reduced application costs and improved farm safety have been observed (Shewry et al., 2008). Traits giving resistance to four different herbicides have been developed: glyphosate, oxynil herbicides, glufosinate herbiciedes and imidazolinone (Agbios Databse, www.agbios.com). Glyophosate-tolerant soybean was the first GM herbicide-tolerant plant commercially grown (Padgette et al., 1995); it contains the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, isolated from the common soil bacterium *A. tumefaciens*, which confers tolerance to glyphosate, the active ingredient in the herbicide Roundup®. Nowadays several GM species containing the EPSPS transgene are available on the market (i.e. sugar beet, canola, cotton, alfalfa, wheat and maize). Pest resistant crops, which are also usual, were developed by

introducing *cry* genes from the soil bacterium *Bacillus thuringensis* (de Maagd et al., 1999). This trait has been successfully introduced into many commercialised crop species including cotton, tomato, potato and maize (Agbios Databse, www.agbios.com). Far from reaching the importance of herbicide tolerant and insect resistant crops, several GM crops resistant to virus are also present on the market.

On the other hand, a few authorised GM crops have improved nutritional characteristics (i.e. canola and soybean with modified seed fatty acid contents and maize with altered amino acid composition) and some plants have enhanced processing quality (i.e. tomato and melon with increased shelf-life and maize expressing a thermostable alpha-amylase). More recently, stress has been placed on the main storage products: starch, oils and proteins. A well-known example is the Amflora potato, approved for cultivation on February.2010 by the EU Commission (http://www.gmo-compass.org) in which amylose and amylopectine compositions have been altered. In fact, the gene encoding the granule bound starch synthase (GBSS) enzyme was inactivated by means of antisense technology. Thus, this potato produces starch basically consisting on amylopectina: this saves an expansive separation step and represents an advantage to the starch processing industries.

Although GM plants used for phytoremediation purposes have not yet reached the market, they have aroused scientific interest over the last decade. Phytoremediation is the use of plants to extract a wide range of heavy metals and organic pollutants in water, sediments or solids. In nature over 400 species have been identified as hyper-accumulators (i.e. high metal bioconcentration factors) (Baker and Brooks, 1989), however efforts in biotechnology are focused on obtaining plants with fast growth and hyper-accumulation in order to speed up the decontamination process. To that end, *Nicotiana glauca* (with high biomass and tolerant to a wide range of environmental conditions) (Gisbert et al., 2003) and the fast-growing grey poplar tree (Peuke and Rennenberg, 2005) have been transformed for phytoremediation applications.

In the last few years, GM lines that combine more than one single trait have gained a great importance. They include insect resistance and herbicide tolerance and/or two different insect resistance modes of action. These so-called stacked gene lines have been developed by crossing single trait GM lines.

Alternatively, simultaneous insertion of multiple genes is a current development in genetic engineering with applications in the food sector and new plants with more complex alterations of metabolic routes are now in progress. The most well-known example is Golden Rice, engineered to synthesise and accumulate pro-vitamin A (β-carotene) in the endosperm. It was developed using *Agrobacterium*-mediated transformation to introduce three required enzymes to complement the synthesis of β-carotene: phytoene synthase gene (PSY) from *Narcisus pseudonarcissus*, phytoene desaturase gene from *Erwinia uredovora* and lycopene β-cyclase from *Narcisus pseudonarcissus* (Ye et al., 2000). In 2005, it was improved by replacing the daffodil PSY with the maize PSY gene. Such modification generated the Golden Rice2, with higher β-carotene contents (Paine et al., 2005). These new plants with improved

nutritional proprieties are often referred to as the 'second generation' of GMOs (Robinson, 2002). Other examples are: oil crops with enhanced seed tocopherol (Karunanandaa et al., 2005), increased vitamin C contents in maize (Chen et al., 2003), augmented available amounts of iron in rice (Lucca et al., 2006), and soybean with accumulated non-host polyunsaturated fatty acids (PUFAs) (Chen et al., 2006).

2.3. Plants as biofactories

The development of the so-called 'third generation' of GM plants is now in progress. In fact, the use of GM plants as biofactories through transformation with genes of pharmacological, industrial or agricultural interest is nowadays a real possibility, which is presumed to be of great interest in the future. It offers a number of possibilities that are especially relevant to the production of proteins with clinical or veterinary applications that have a high added value. Although no plant-made vaccines and biopharmaceuticals have entered the market to date, great research advances have been achieved in the last two decades (for more details see reviews Boehm, 2007; Daniell et al., 2009). Recent examples of plant produced proteins that have had clinical trial success are a vaccine for non-Hodgkin's lymphoma (McCormick et al., 2008), a protein-based therapeutic (IFN-α2b) to combat hepatitis C (http://biolex.com) and a human glucocerebrosidase to combat Gaucher's disease (Aviezer et al., 2009) (for a review, see Daniell et al., 2009).

Related to compounds with industrial interest, the trend of higher demand for polymers and chemicals obtained by renewable resources opened a new door for transgenic plants as a platform to produce them. Just as an example to show the potential of this technology: fibrous proteins such as silk and collagen were produced in *Arabidopsis* (Ruggiero et al., 2000; Yang et al., 2005); polyhydroxyalkanoates (PHAs, potential replacement for polypropylene and other petrochemical polymers) have been synthesised in a range of plant species including oilseed rape (Houmiel et al., 1999), sugar beet (Menzel et al., 2003) or sugar cane (Petrasovits et al., 2007). Moreover, this group of compounds with industrial applications also includes enzymes with industrial interest, such as cellulases required to convert lignocellulose to liquid fuel. Recently the expression of cellulase and xylanse genes in maize with activation of the enzyme in the plant after harvesting has been reported (Biswas et al., 2006; Ransom et al., 2007).

In the frame of agricultural applications, the existence of peptides and small proteins exhibiting direct antimicrobial activity is well known, these have great perspectives as a new class of phytosanitary compounds for plant disease protection. Biotechnology companies such as Maltagen Forshung GmbH, Meristem Therapeutics, Saponin Inc. and Ventria Bioscience have ongoing projects to produce the antimicrobial lactoferrins using plant biofactories based on barley, corn, tobacco, potato, rice and saponaria (Spök et al., 2006).

In any case, among other advantages of GM plant biofactories are strategies based on constitutive and conditional promoters, including tissue-specific; the possibility of targeting the heterologous protein to different cell compartments [which may in turn reduce its

cytotoxicity and increase its stability, (Conrad and Fiedler, 1998; Takagi et al., 2005)]; the scalability, sustainability and safety as "green" (instead of "chemical") production, greenhouse containment and self-pollinating crops and/or vegetative reproduction; the possibility of introducing other transgenes through sexual crossings; the high yield reported in some cases (1% total protein) and the relatively low product recovery costs (for certain proteins and applications, which is especially relevant when plants can directly be used).

3. Regulatory framework for GMOs in EU

Since the introduction of the first GM plant varieties on the European market in 1996, the EU has established a legal framework in order to ensure that not only the development of this modern technology takes place in complete safety but also its release into the environment and its commercialisation.

3.1. Current GMO legislation

Currently, two Directives are the main legislation which governs the contained use of GMOs (Council Directive 98/81/EC) and their deliberate release into the environment (Directive 2001/18/EC). They repeal the earlier Council Directive 90/219/EEC and Council Directive 90/220/EEC respectively, which had been in place since 1990s. The new Directive 2001/18/EC tightened up on the authorisation procedure of transgenic organisms in order to improve the efficiency and transparency of the process. In this way, the Directive provides a step by step process for risk assessment, that is, a common methodology to identify and evaluate potential and adverse effects of the GMO on human health and the environment. Moreover, it limits permits to 10 years and requires monitoring of the impact of GMOs on the environment. For GMO risk assessment, the European Food Safety Authority (EFSA) plays a crucial role.

The progressive increase of biotech area has given rise to a situation of coexistence, which has been regulated in Europe since 2003 (Commission Recommendation No 2003/556/EC). Coexistence is understood as the ability of farmers to make a practical choice between conventional, organic and GM-crop production, in compliance with the legal obligations for labelling and/or purity standards. According to this document measures for coexistence should be developed and implemented by the Member States.

Related to GM food and feed, Regulation (EC) No 1829/2003 covers the Community procedures for its authorisation and supervision as well as instructions for labelling. This regulation is stricter than the previous one. It includes all food or feed which consists of, contains or is produced from GMOs (containing or not, DNA or protein resulting from genetic modification). It establishes a 0.9% threshold for compulsory food labelling, replacing the original 1% (Commission Regulation (EC) No 49/2000). There is also a 0.5% threshold for GMOs not approved for use in the EU that have received a favourable scientific risk assessment. The requirement of a method for detection, sampling and identification of the

transformation event is part of the new authorisation procedure. These methods should be validated by the European Commission Joint Research Centre (JRC) which was established by the Regulation (EC) No 1829/2003 as new Community Reference Laboratory (CRL) and is supported by the European Network of GMO Laboratories (ENGL). Commission Regulation (EC) 641/2004 and Commission Recommendation No 784/2004 give detailed rules for the implementation of Regulation (EC) No 1829/2003, in particular, the first one provide guides to fulfil all requirements in the authorisation process and the second one offers technical guidances for sampling and detection. Finally, Regulation (EC) No 1830/2003 lays down rules on traceability and labelling of GMOs and products produced from GMOs. It amends Directive 2001/18/EC requesting Member States to take measures to ensure traceability and labelling of authorised GMOs at all stages of their placing on the market.

3.2. Critical aspects in GMO regulation

3.2.1. Authorisation procedure

According to EU legislation (Regulation (EC) No 1829/2003 and Directive 2001/18/EC) a GMO can only be introduced in the market or be deliberately released into the environment if it can be documented as safe. The starting point of biosafety evaluation is the precautionary principle: a case by case human health and environmental risk assessment must be carried out prior to any release.

Although this principle is common for the commercial use of all GMOs, the regulation is not the same for GM products that will be processed prior to use and GM products that can be propagated and cultivated. In the first case the authorisation process is based on the GM food and feed regulation (Regulation (EC) No 1829/2002). The application form should be accompanied by molecular characterisation, chemical comparative analysis showing that the GM food is substantially equivalent to conventional counterparts, food/feed safety assessment, environmental risk assessment and post-market environmental monitoring plan. It shall be submitted to EFSA, and EFSA will return the European Commission (EC) and the Member States an official opinion based on the scientific evaluation by a panel of experts (the GMO panel). Finally, the EC will produce a decision.

On the other hand, the authorisation procedure for GM products that can be propagated and cultivated is based on the directive for the deliberate release of GMOs into the environment (Directive 2001/18/EC). The basis for this authorisation is an environmental risk assessment (era). In this case, the application is submitted to a Member State which performs an initial assessment and emits a scientific opinion to the EC. In the case of objections a safety assessment from EFSA can be required. If objections are favourably resolved, the competent authority (EC and/or Member States) authorises the product and it can be placed on the market throughout the EU. In spite of this, Member States can invoke a so called 'safeguard clause' (Directive 2001/18/EC) to provisionally restrict or prohibit the use or sale of the GM product on its territory.

The regulatory framework for cultivation and commercialisation of GMOs has evolved from 1996 to date to get adapted to the public worries and requirements. It has contributed to overcome the '*de facto*' moratorium concerning the marketing of new GMOs that was established in October 1998 for five years. However, one of the main consumers' reservations about the impact of GMOs on human health is the incidence of possible unintended alterations in their composition (Kuiper et al., 2004).

3.2.2. Labelling

As it has been previously described, new and strict regulations (Commission Recommendation No 784/2004/EC; Regulation (EC) No 1829/2003; Regulation (EC) No 1830/2003) give instructions for GMO labelling. Labels are often considered as warnings for the consumers who erroneously believe that they are meant to notify health hazards. In fact, a commercialised GMO has been approved as safe; consequently labelling was introduced to give consumers the freedom of choice (i.e. allowing the decision between products from different agricultural systems). During production, transport and processing admixture of small amounts of crops from different origins is considered unavoidable. Thus, the labelling requirement sets a threshold for accidental presence of GMOs which attempts to be an equilibrium between producers' requests and consumers' requirements.

To comply with the labelling regulations, but also for traceability and coexistence purposes there was a need of analytical tools that allow reliable identification and quantification of specific GMO events. Basically we can distinguish bioanalytical methods (including DNA-based and protein-based techniques), chemical methods and bioassays; the choice depending upon the particular sample and circumstances. Polymerase chain reaction (PCR) is accepted as the reference technique for regulatory compliance in the EU. A general GMO detection is possible by targeting screening elements or construct-specific sequences. GMO event specific detection can be performed by real-time PCR (qPCR) based amplification of transgene and host plant flanking sequences. GMO quantification requires parallel analysis of species specific reference genes. A number of reviews covering GMO analytical tools are available (Pla et al., 2010; Miraglia et al., 2004). In view of the increasing number of GMOs in the global market, multiplex approaches are quickly evolving. Examples are multiplex PCR coupled to detection strategies such as capillary gel electrophoresis (CGE) (García-Cañas et al., 2004; Nadal et al., 2006; Nadal et al., 2009) or array format technologies (Hamels et al., 2009; Leimanis et al., 2006; Rønning et al., 2005); or nucleic acids based amplification (NASBA) implemented microarray analysis (NAIMA) (Morisset et al., 2008). For high-grade multiplexing analysis approaches based on PCR amplification with universal primers and detection by hybridisation on array supports showed to be effective (Chaouachi et al., 2008; Prins et al., 2008). Note that most multiplex tools are not quantitative and they are not limited to event specific target sequences. A qualitative analysis to test whether GMO(s) are present in a given sample and the identification of specific GMO event can be initially performed by a matrix approach. If needed, subsequent quantification can be carried out.

Despite the huge progress, GMO analysis still poses some interesting challenges. A report from the Commision on the implementation of Regulation (EC) No 1830/2003 (European Commission Report, 2008) expressed the difficulty in inspecting stacked events and unauthorised GMOs. Although some of the mentioned high-throughput methods have the potential to detect unknown GMOs, their detection is not always possible with the currently applied screening strategies; consequently it is envisaged that more progress will be achieved in this sense in the near future.

3.2.3. Traceability

According to Regulation (EC) No 1830/2003 traceability means the ability to trace GMOs and products derived from GMOs at all stages of their placing on the market through production and distribution chains. The general objectives of traceability are: to facilitate GMO labelling, to monitor possible potential effects on the environment and on health and to recall from the market a product if an unexpected risk to human health or to the environment is detected.

Traceability involves that anyone who introduces a GM product in the market must provide information on the unique identifier for this GMO to those who are next in the supply chain. Moreover, for a period of five years, every operator must keep this information and be able to identify the operator from whom the product is obtained and to whom it is supplied.

3.2.4. Coexistence

The European regulation established the concept of coexistence as 'the principle that farmers should be able to cultivate freely the agricultural crops they choose, be it GM crops, conventional or organic crops'. Currently there is not an EU wide-legislation on the coexistence of GM crops and all European countries need to develop national strategies to ensure coexistence (Commission Recommendation No 2003/556/EC).

According to a report from the EC on the coexistence of GM crops (European Commission Report, 2009), since 2006 Member States have made significant progress developing coexistence legislation. In 2009, 15 Member States adopted specific legislation on coexistence and the majority has designed coexistence measures to prevent adventitious presence of GMOs above the labelling threshold for GMOs in food and feed. Twelve Member States adopted segregation measures for at least one crop, although they vary considerably. As an example, isolation distances of GM and conventional maize range between 25 m and 600 m. Isolation distances can be replaced in some cases by buffer zones between GM and non-GM fields, however in other Member States buffer zones are mandatory supplements. Moreover, the possible use of different flowering times is only allowed in two Member States. Research activities in this area have been carried out in order to evaluate the adventitious presence of GMO in non-GM fields and to evaluate different tools to reduce it (Messeguer et al., 2006; Palaudelmas et al., 2008; Palaudelmas et al., 2009a). In some cases there is a lack of harmonisation between the scientific results and the segregation measures proposed by Member States. Regarding differences in coexistence approaches applied by Member States, the Commission plans to take several actions (e.g. to develop technical guidance

documents, to support further research and to foster an exchange of information within Member States) (European Commission Report, 2009).

4. Cultivation and commercialisation of GM crops

4.1. Current worldwide acreage for GM crops

The first GM plant varieties appeared on the United States' market in 1994; they were tomato varieties modified to slow down the ripening process, so they had longer shelf life. Tomato is an important fruit crop but it was the release and success of GM varieties of two of the major agricultural crops, soybean and maize, that really established genetic modification as an important tool in plant breeding. These varieties were first grown in a large scale in the USA in 1996.

Since then, the global transgenic area grew from 1.7 in 1996 to 134 million hectares in 2009. Compared to 2008, there was a growth rate of 7% and overall, the global GM crop area has increased approximately 80-fold since 1996 (James, 2010).

In 2009, the number of countries planting biotech crops reached the figure of 25, comprising 15 developing countries and 10 industrial countries. The top eight countries growing more than one million hectares each were (in decreasing order by hectarage): USA (64 million hectares), Brazil (21.4), Argentina (21.3), India (8.4), Canada (8.2), China (3.7), Paraguay (2.2), and South Africa (2.1). The remaining 17 countries which grew biotech crops in 2009 were (in decreasing order by hectarage): Uruguay, Bolivia, Philippines, Australia, Burkina Faso, Spain, Mexico, Chile, Colombia, Honduras, Czech Republic, Portugal, Romania, Poland, Costa Rica, Egypt and Slovakia (James, 2010).

The major GM crop was soybean, occupying 52% of global biotech area in 2009; followed by maize (31%), cotton (12%) and canola (5% of the global biotech crop area). In reference to traits, from the beginning of commercialisation in 1996 to 2009 herbicide tolerance has consistently been the main trait. In 2009, herbicide tolerance deployed in soybean, maize, canola, cotton and alfalfa occupied 62% of the global biotech area. For the third year, in 2009 the stacked double and triple traits occupied a larger area (22% of global biotech crop area) than insect resistant varieties (15%). Insect resistance products were the fastest growing trait group between 2008 and 2009 at 14% growth whilst stacked trait and herbicide tolerance products grew at the same rate of 6%. In 2008 a new biotech crop, RR® herbicide tolerant sugar beet, was introduced in USA and Canada, which became the fastest adopted biotech crop globally in 2009 (James, 2010).

A remarkable feature of the global status of GM crops is the rapid and enthusiastic uptake of GM varieties in some countries and the resistance to GM crops in other countries, notably in the EU where only the insect resistance maize (MON810) is authorised for cultivation. Just six out of the 27 countries in the EU officially planted MON810 maize on a commercial basis

in 2009. They were (listed from higher to lower hectarage): Spain, Czech Republic, Portugal, Romania, Poland and Slovakia. All of them increased their MON810 maize hectarage in 2008; however hectares changes between 2008 and 2009 varied and only Portugal reported higher Bt maize hectarages (James, 2010). Despite the opposition by some consumers and environmentalists, in Spain maize area decreased in 2009, but the highest percentage of GM maize (21.8%) was recorded according to official data from the Spanish government (MARM, Gobierno de España, www.marm.es). Similarly, in Catalunya the biotech area has consistently increased at a high rate (Figure 2) reaching a proportion of 51% of total maize in 2009 (DAR, Generalitat de Catalunya, www.gencat.cat).

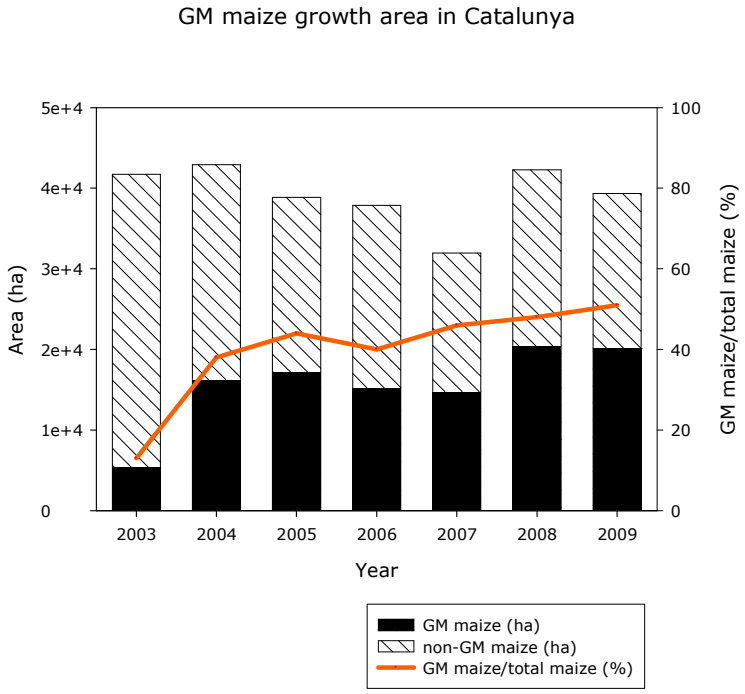

Figure 2. Evolution of total GM maize growth area in Catalunya from 2003 to 2009. Horizontal bars show the evolution of GM maize (black) and non-GM maize (patterned) hectarage cultured in Catalunya. The orange line plot represents the evolution of the contribution of GM maize expressed as % of total maize area (DAR, Generalitat de Catalunya, www.gencat.cat).

4.2. The particular case of MON810 maize

MON810 maize (Monsanto, USA - Yieldgard®) was approved under Council Directive 90/220/EEC for growing, import, seed production and processing into animal feeding stuffs and industrial purposes on April the 22nd of 1998 (Commission Decision 98/294/EC). On June 2009 the EFSA emitted a favourable scientific report concluding that maize MON810 is as safe as conventional maize in response to the application for renewal of its authorisation (EFSA, 2009a).

MON810 was engineered to be resistant to certain lepidopteran target such as the European corn borer (*Ostrinia nubilatis*) and species belonging to the genus *Sesamia*, major insect pests in agriculture. Caterpillars of these lepidopteran insects damage stalks, chewing

tunnels that cause plants to fall over and in consequence, its yield decreases. They also damage maize ears, which has been correlated to increase mycotoxin levels in grains (Serra et al., 2006).

MON810 maize was developed by transfer of a synthetic gene encoding a truncated version of the insecticidal protein CryIA(b), derived from *Bacillus thuringensis*, into the maize cultivar Hi-II by biolistic transformation. CryIA(b) is one of the Cry delta-endotoxins. It is specifically active against specific lepidopteran insects and no mammal species have been reported as targets (Federici, 2002). Once activated by insect digestive proteases (Pigott and Ellar, 2007), CryIA(b) selectively binds to specific receptors localised on the brush border midgut epithelium and cation-specific pores are formed which disrupt midgut ion flow causing epithelial cell death and subsequent septicaemia and death of the larvae (Jiménez-Juárez et al., 2007; Lemaux, 2009).

The introduction of the *cryIA(b)* transgene was performed by using two plasmids: PV-ZMBK07 and PV-ZMGT10. The first contained the *cry1Ab* gene under the control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter (e35S-P) and the nopaline synthase 3' terminator signal from *A. tumefaciens* (Nos-T). An intron from the maize heat-sock protein gene (*hsp70*) preceded *cry1Ab* to regulate its expression. PV-ZMGT10 contained the CP4 *epsps* gene (from *Agrobacterium* sp.) which allowed selection through glyphosate tolerance; and the *gox* gene (from *Ochrobactrum anthropi* sp.) which encodes a glyphosate metabolising enzyme. They were also regulated by e35S-P and NOS-T. Both plasmids contained the *nptII* gene as bacterial selectable marker (EFSA, 2009a).

Molecular characterisation of maize MON810 revealed that it contains a single transgene insertion which only consists of elements derived from plasmid PV-ZMBK07, including the e35S-P, the maize *hsp70* intron and part of the *cryIAb* coding sequence (EFSA, 2009a). The data indicated that no other portions of this plasmid and no portions of plasmid PV-ZMGT10 were present. Thus, no reporter genes are present in this event. Moreover, a short portion at the 3' end of the *cry1Ab* gene as well as the NOS-T have been deleted probably due to genomic rearrangements during the integration process (Hernández et al., 2003). A recent study identified the 3' genomic junction as an intron of a truncated gene coding for the HECT E3 ubiquitin ligase in the reverse orientation (Rosati et al., 2008). Expression analysis of *cryIAb* indicated the transcription read-through 3' past the *cryIA(b)* sequence and the existence of different lengths of transgene transcripts (La Paz et al., 2010; Rosati et al., 2008). In addition, the presence of a Stop codon at position +7 downstream the truncation site drove to the production of transgenic CryIA(b) protein with only two aminoacids additioned after the truncation point. The DNA sequence 5' flanking the insert MON810 is homologous to a LTR sequence of the *Z. mays* 22kDa *α–zein* gene (Holck et al., 2002).

In maize, the number of genotypes suitable for transformation and effective plant regeneration is still limited (Zhang et al., 2004). Thus, the MON810 transgene was introduced into a maize line with good plant regeneration efficiency but very little agronomic value. Backcrossing programs (with lines with commercial interest) are then applied by seed

companies to develop a line that contains the transgene insert in the desired genetic background (Figure 3). In other words, the process results in a transgenic line carrying the transgene (together with a small portion of the originally transformed genome) in a genetic background almost exclusively belonging to other line(s) of commercial interest. Commercial maize seeds are hybrids i.e. they are obtained by crossing two inbred lines (Cereal Knowledge Bank, http://www.knowledgebank.irri.org). Actually hybrid lines are uniform in appearance, competitive with weeds and have high yield, based on the hybrid vigour. To obtain commercial transgenic seeds, the inbred line carrying the transgene is crossed with another inbred line. This way, seed companies have their own transgenic varieties.

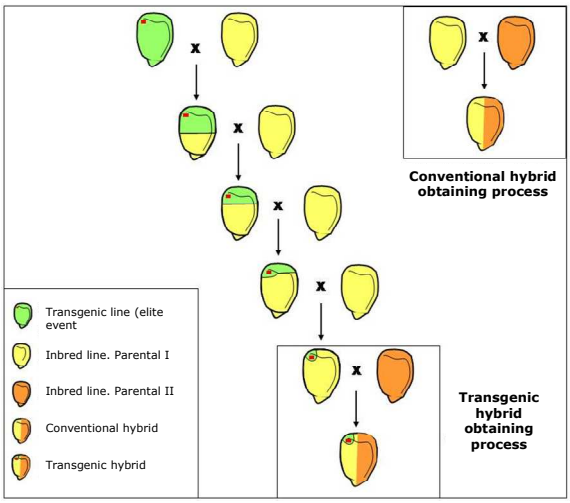

Figure 3. Conventional and transgenic hybrid production process (extracted from Palaudemàs, 2009b).

The non-GM variety the most closely related to a given GMO is here termed near-isogenic. GMO and near isogenic variety pairs have similar agronomic characteristics. The process and the precise lines used to obtain a commercial GM variety are treated by seed companies as confidential. Thus, it is not possible to know the real genetic relationship, the exact genetic distance between a given GMO and the non-GM counterpart: the companies only state they are closely related. Isogenic lines are those genetically identical (except for sex), obtained from a single individual or from the same inbred strain. Near-isogenic lines can be defined as 'lines that are identical except at one or a few genetic loci' (www.nature.com/nrg/journal/v4/n11/glossary/nrg1206_glossary.html); or 'lines that have the same background with different relatively small proportions of the donor parent genome' (Szalma, 2007). In other instances, null segregants from a transformation protocol have been considered near-isogenic to these transgenic lines (FAO, 2004). It should be noted that in maize the near-isogenic line to a GMO is not 'a non-GM line with identical genome with the only exception of the transgene' but it has a certain (unknown) amount of other genomic differences.

The MON810 is actually commercialised in many different varieties, with different genomic backgrounds making it adapted to different climatic conditions and meant for different

purposes (e.g. food or feed). As an example, in 2009, 123 MON810 commercial varieties were inscribed in the Spanish *Registro de Variedades Vegetales* and could be cultured in Spain (EFSA, 2009).

5. Safety assessment of GM crop derived foods

As has been mentioned, GM crops are subjected to different legislation worldwide to cover aspects of consumer safety and protection. From the first initiatives to establish a global framework to assess the safety of GM crops, the Principle of Substantial Equivalence has been the leading principle. A critical element in this principle is the detection of potentialy unexpected differences between GMOs and non-GM comparable lines, which is the main subject of this thesis.

5.1. Principle of Substantial Equivalence

The Principle of Substantial Equivalence is a comparative approach which assumes the safety of traditional crops based on their history of use; consequently, they are taken as reference to assess GM crops. This approach has been further developed by the Organisation for Economic Co-ordination and Development (OECD, 1993) and The United Nations World Health Organisation/Food and Agricultural Organisation (FAO/WHO, 2000). It is the starting point for food safety evaluation (Kok and Kuiper, 2003). Actually, the process includes compositional analysis (e.g. nutrient, anti-nutrient, allergens and natural toxins) and comparison of phenotypic and agronomic characteristics. In case differences between a GMO and the non-GM counterpart are observed, further toxicological and nutritional studies must be carried out. According to this concept, OECD, FAO/WHO and International Life Sciences Institute (ILSI) defined three possible scenarios to typify novel GM crops:

- Substantially equivalent to the comparator: no further analyses are required.
- Substantially equivalent to the comparator except for the transgene: additional analyses focusing on the transgene are necessary (e.g. a specific toxicity and allergenicity test of the new protein, potential gene transfer and role of the novel product in the diet).
- Not sufficiently equivalent: a case-by-case assessment of the new product is required.

According to some authors (Cellini et al., 2004; König et al., 2004) there are several critical points which have to be taken into consideration to ensure successful application of this principle:

- Availability of a comparator: while the ideal comparator of a GM line is the direct parental line, it is not always available. Commercial GM crops are commonly obtained by crossing a specific male pollinator line (non-GM marketed crop) with the transformed line to obtain the GM cultivar. In that case, the best comparator

should be the null segregant from the protocol transformation that has identical background while lacking the transgene. However, it is only available to the companies that have developed the GM line. Thus, comparison of GM and available non-GM crops may show differences unrelated to the genetic modification, so comparison to several relevant lines is recommended.

- Selection of compounds: current compositional analyses are based on targeted approaches to compare GM and its non-GM counterpart; consequently, results of the comparison will depend on the selected compounds. The OECD formulated consensus documents presenting the elements that should be analysed on individual crop plants (OECD, 2003); consensus documents on canola, soybean, potato, sugar beet, maize, bred wheat, rice, cotton, barley, sunflower and tomato have been published (OECD, http://www.oecd.org). Additional analyses are required depending on the type of genetic modification, which should be decided on a case-by-case basis.
- Discrimination between differences in the GM/non-GM crops that outcome from the genetic modification and those differences introduced during tissue culture (somaclonal variation) and/or environmental conditions. To limit the misinterpretation of differences caused by environmental conditions it is necessary to grow GM and comparable plants under the same conditions and under a range of environments and climates (Kok and Kuiper, 2003).

Summarising, the Principle of Substantial Equivalence is a tool to look for differences between GM crops or derived food and their comparator and has been proposed as the most suitable approach for risk assessment (FAO/WHO, 2000). However, it has been criticised considering that the current approach used to compare GM crops to their non-GM counterparts is only based on targeted analyses, so it is biased; and further it does not take into consideration possible unintended effects due to the biotechnological process (Millstone et al., 1999).

5.2. Unintended effects: definition

Unintended effects were defined by Cellini and co-workers (Cellini et al., 2004) as statistical significant differences between a GM plant and its comparator, a part from the new gene introduced. They can be predictable (when the effects are expected and explicable in terms of the present knowledge on plant metabolism and physiology) or unpredictable (which are changes falling outside our present level of understanding).

Although unintended effects resulting from conventional breeding are significant, in the last years this concept gained importance in relation to the application of recombinant DNA technology. The integration of a transgene in the genome's plant is a random process with preference for gene-rich regions (Koncz et al., 1992), so disruption, modification or silencing of active genes and production of new proteins might occur (transgene position effects). In addition, pleiotropic effects could arise as a result of transgene products interacting with the

regulation of other genes or the activity of other proteins (Miki et al., 2009). Unintended effects can be positive, negative or null but they have to be taken into account.

5.3. Unintended effects: identification by profiling methods

The use of targeted analysis to assess the safety of new GM crops implies the selection of a limited number of compounds to be analysed. Therefore, unintended effects, and specifically, unexpected effects could remain undetected. Profiling methods permit to screen for possible differences at different cellular levels: gene expression (transcriptomics), proteins (proteomics) and metabolites (metabolomics); consequently, they have been proposed as a complementary safety assessment tool in this frame (Cellini et al., 2004; FAO/WHO, 2000; König et al. 2004; Kuiper et al., 2001). Their usefulness has been demonstrated by the numerous recent publications reporting the use of profiling technologies to evaluate possible unexpected effects of various transgenes in several plant species (Table 1).

Table 1. Profiling techniques for unintended effects detection in GM plants

Host plant	Inserted gene	Trait	Approach*	Reference
	nptII	Marker gene		
Arabidopsis	*nptII* and GUS	Marker + reporter gene	T	El Ouakfaoui et al., 2005
	HPH and GUS-GFP	Marker + reporter gene		
Arabidopsis	CYP79A1, CYP71E1, and UGT85B1	Dhurrin biosynthesis	M	Kristensen et al., 2005
Arabidopsis	GUS	Reporter gene	P	Ruebelt et al., 2006b
	hppd and γ-tocopherol-methyltransferase,	Tocopherol enhanced		
Arabidopsis	bialaphos resistance	Glufosinate tolerance	T	Abdeen et al., 2009
Arabidopsis	*bar*	Reporter gene	P	Ren et al. 2009
Arabidopsis	transcription factor ABF3	Drought tolerance	T	Abdeenet al., 2010
maize	cry1Ab (MON810)	Insect resistance	M	Manettiet al., 2006
maize	cry1Ab (MON810)	Insect resistance	M	Levandi et al., 2008
maize	cry1Ab (MON810)	Insect resistance	P	Zolla et al., 2008
maize	cry1Ab (MON810)	Insect resistance	T	Coll et al., 2008 (this thesis)
maize	cry1Ab (MON810)	Insect resistance	M	Piccioni et al., 2009
maize	cry1Ab (MON810)	Insect resistance	T,P,M	Barros et al., 2010
	EPSPS (NK603)	Glyphosate tolerance		
maize	cry1Ab (MON810)	Insect resistance	P	Coll et al., 2010b (this thesis)
maize	cry1Ab (MON810)	Insect resistance	T	Coll et al., 2010a (this thesis)
	sucrose phosphorlase	Modified in sucrose metabolism		
potato	invertase	Modified in sucrose metabolism	M	Roessner et al., 2001
	glucokinase	Modified in sucrose metabolism		
potato	sucrose:sucrose 1-fructosyl-transferase	High levels of inuli-type fructans	M	Catchpole et al., 2005
	fructan:fructan 1-fructosyl-transferase	High levels of inuli-type fructans		
	W2	Glucan-branching enzyme		
potato	*Mal1*	Alpha-glucosidase activity	P	Lehesranta et al., 2005
	S-adenosylmethionine decarboxylase	S-adenosylmethionine decarboxylase		
potato	aprotinin	Serine protease inhibitor	P	Badri et al., 2009
potato	cathepsin D inhibitor	Insect-resistance	P	Goulet et al., 2010
potato	cathepsin D inhibitor	Insect-resistance	P	Khalf et al., 2010
rice	anthranilate synthase alpha subunit	Accumulate large amounts of free tryptophan	T,M	Dubouzet et al., 2007
rice	ScFv antibody against carcinoembryonic antigen	Biofactory for ScFv antibodies	T	Batista et al., 2008
rice	cryIAc and sck	Insect resistance	M	Zhou et al., 2009
soybean	EPSPS	Glyphosate tolerance	T	Cheng et al., 2008
tomato	nucleoprotein gene of tomato spotted wilt virus	Virus resistance	P	Corpillo et al., 2004
wheat	phytase	Improved hydrolisis of phytic acid	T	Gregersen et al., 2005
wheat	HmW glutenin gene subunit 1Ax1	Food processing applications	M	Beale et al., 2009
wheat	HmW glutenin gene subunit 1Ax1	Dough strengh	T	Baudo et al., 2006; 2009

*T: transcritpomics; P: proteomics; M: metabolomics

5.3.1. Transcriptomics

Overall gene expression analysis can be performed using the microarray technology. It is a hybridisation-based method derived from the precursor technique Northern Blot but allowing simultaneous determination of the expression levels of a large number of genes. In microarray hybridisation a labelled sample hybridises in a specific way to probes immobilised

on a solid support. Messenger RNAs (mRNAs) extracted from biological samples are labelled and physically separated on the array according to the position of their target probe, which enables the individual quantification of many specific mRNAs in a single hybridisation experiment. Moreover, the use of different dyes possessing different excitation and emission characteristics allows simultaneous analysis of two different samples (control and test sample). Normalised data gives the relative expression level for each gene in the sample investigated.

According to the manufacturing method, two main microarray types can be distinguished. Spotted arrays are custom fabricated microarrays that use printing-type technologies which are based on the deposition of previously synthesised sequences onto a solid surface. They gather in two classes: contact printing (mechanical methods) and non-contact printing (ink-jetting systems). Usually double-stranded DNA (dsDNA) [complementary DNA (cDNA) or PCR product ranging in size from 250 to 1000 bp] or long synthetic oligonucleotides (30-70 mer) are spotted as probes (Mir and Southern, 1999). The second type of manufacturing methods is the so called in-situ synthesis systems which use short oligonucleotides (20-25 mer) in a high-density format. Within this group, the most widely used method is the photolithographic manufacturing process (e.g. Affymetrix, Santa Clara, CA; Agilent, Palo Alto, CA). Its principle is based on a glass substrate modified with covalent linker molecules containing photolabile protecting groups. In each manufacturing step the solid substrate is selectively activated and flooded with a modified nucleotide which will be coupled at the activated region of the chip (Pease et al., 1994).

Labelling and hybridisation are the first step in a microarray experiment. Single-channel (a single sample at a time) or multiple-channel (two or more samples simultaneously) arrays can be used depending on the number of samples that are processed on one array. An example of single-channel arrays are Affymetrix GeneChips whose labelling procedure involves: in vitro synthesis of biotinylated anti-sense copy RNA (cRNA), fragmentation and hybridisation of the cRNA, and chips labelling with streptavidin phycoerythrin conjugate. Incubation with antibody anti-biotinylated streptavidin allows signal amplification. One of the main benefits of this type of microarray is that data from different experiments can be easily compared. Alternatively, using a multiple-channel array, mRNA is fluorescent labelled by first-strand cDNA synthesis. A mixture of equal amounts of control and test samples labelled with different fluorescent dyes (e.g. Cyanine-3 and Cyanine-5) is hybridised in a competitive way to the array. In any case, after hybridisation, unbound material is washed away and the hybridised sample is visualised by fluorescence detection.

Different companies are currently commercialising arrays (including chips, reagents and software). Maize commercial Affymetrix chips (GeneChip® Maize Genome Array) were used in this work, which allow simultaneous analysis of 17,555 probes, representing 13,339 genes, i.e. around 1/3rd of the genes of maize (Schnable et al., 2009).

Fluorescence data has to be pre-processed to obtain the expression level of each gene in the sample tested. To that aim, different methods are available depending on the array type:

single-channel (gives relative expression levels) or multiple-channel arrays (from which absolute expression levels are obtained). In the second case, the most used pre-processing method is the so called Robust Multi-array Average (RMA) which includes background correction, normalisation and summarisation. Basic transcriptomic experimental designs are based on two-group comparisons; using fold changes and T-test analysis to identify differentially expressed genes. The main drawback of T-test statistics applied to microarray data is the high occurrence of false positive results due to the very high number of simultaneously tested genes. Multiple testing correction methods such as Bonferroni or Benjamini and Hochberg False Discovery Rate (FDR) have to be applied in order to preserve the global error rate. On the other hand, microarray experiments based on multiple comparisons generate large amounts of data that can be more easily interpreted using several multivariable statistic techniques such as principal component analysis (PCA), self-organizing maps (SOM) and hierarchical clustering.

Nowadays, gene expression analysis is the most extensible application of microarray technology. Schena and colleagues (Schena et al., 1995), showed for the first time the usefulness of this technique to compare the expression level of 45 genes in two different tissues of *Arabidopsis*. Since then, numerous experiments have been performed in several plant species to study the effects of different factors (e.g. different tissues, developmental stages, environmental conditions). In the last years, microarray technology has also been applied in the field of GMOs as a tool to detect possible unintended effects resulting from DNA recombinant techniques in *Arabidopsis* (Abdeen et al., 2009; Abdeen et al., 2010; El Ouakfaoui and Miki, 2005),, wheat (Baudo et al., 2006; Baudo et al., 2009; Gregersen et al., 2005), maize [(Barros et al., 2010), this thesis (Coll et al., 2008; Coll et al., 2010a)], rice (Batista et al., 2008; Dubouzet et al., 2007) and soybean (Cheng et al., 2008).

5.3.2. Proteomics

Proteomic techniques study global protein accumulation in a cell, tissue or organism in a specific moment and have been proposed as valuable analytical tools to complement current safety assessment techniques (FAO/WHO, 2002). In fact, they are tools of special interest because they could allow the identification of known toxins, antinutrients or allergens (in case of suspition of a new allergene, etc. it should be assessed by other type of studies such as epidemiological). The most extensively used approach is based on a two-dimensional electrophoresis gel (2DE) for protein separation and quantification followed by analysis and identification of the differential spots by mass spectrometry (MS).

By coupling isoelectric focusing (IEF) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) the 2DE technology allows protein separation by isoelectric point (pI) in the first dimension and by molecular mass (Mw) in the second dimension (O'Farrell, 1975). Proteins can be then detected using different staining methods, among which coomassie brilliant blue (CCB), silver and fluorescence staining. To detect quantitative changes in protein expression between different species, tissues or conditions specialised softwares for the analysis of 2DE protein profiles have been developed (Nishihara and

Champion, 2002; Pun et al., 1988). Most of them allow spot detection, background correction, gel matching, normalisation, comparison and quantification. Spots of interest are excised from the gel, digested and finally identified by MS.

MS principle is based on ionisation and transference of molecules to be analysed from a condensed phase into a gas phase in order to obtain their molecular mass and their mass-to-charge ratio. Mass spectrometers consist on three modules: ionisation source (generates ions), mass analyser (sorts the ions by their masses by applying electromagnetic fields) and detector (provides data for calculating the abundances of each ion). Several types of mass spectrometers, which can combine different ionisation sources with various mass analysers, are nowadays available. Main ionisation sources applied in the study of biomolecules are electrospray ionisation (ESI) (Fenn et al., 1989) and matrix assisted laser desortion ionisation (MALDI) (Karas and Hillenkamp, 1988); both are especially useful for ionisation of biomolecules because they overcome the propensity of these molecules of excessive fragmentation. The ESI mechanism consists on the dispersion of the liquid containing compounds of interest into multiply charged ions in gaseous phase by electrospray. In contrast, MALDI ionisation is triggered by a laser beam which irradiates a crystalline solution containing the analyte in an organic matrix (which protects biomolecules and facilitates vaporisation and ionisation). Related to mass analysers we can highlight quadripole (Q), capable to analyse a continuous flux of ions; ion trap (IT) and particularly linear IT (LIT) which allow high-throughput analyses with high sensibility; and time of flight (TOF) analyser. They are all compatible with ESI platforms and TOF can operate with both ESI and MALDI. Mass spectrometers can be used to determine not only molecular mass of peptides but also to resolve additional structural characteristics such as amino acid sequence and type of posttranslational modifications in experiments so called tandem mass spectrometry (MS/MS). By means of separate MS steps in space or time, specific ions are selected, subjected to fragmentation through collision and sequenced. There are a number of tandem MS approaches which have different abilities to support specific analytical strategies (see review Domon and Aebersold, 2006). Finally, high specificity and sensibility can be achieved with an on-line reversed-phase liquid chromatography (LC) which concentrate and separate peptides before sequencing by MS (Gygi et al., 2001). The manuscript of this thesis is based on 2DE coupled to LC-ESI-IT.

Besides other multiple applications in plant functional analyses, 2DE-MS technology was used to evaluate possible unintended changes in protein expression and post-translational modifications that occur in GM *Arabidopsis* (Ren et al., 2009; Ruebelt et al., 2006b), tomato (Corpillo et al., 2004), potato (Badri et al., 2009; Goulet et al., 2010; Khalf et al., 2010; Lehesranta et al., 2005) and maize [this thesis (Coll et al., 2010b); (Barros et al., 2010; Zolla et al., 2008),]. Despite of the increasing knowledge of the proteome and the advances in the technology [e.g. immobilised pH gradients (IPGs) which offer greater reproducibility (Blomberg et al., 1995); high sensitive fluorescent strains], this proteomic approach has still important and well-known drawbacks: poor solubility of membrane proteins, limited dynamic range and difficulties in displaying and identifying low-abundance proteins (Lopez, 2007).

Actually, it has been reported that proteomic coverage obtained by 2DE is around 2000 proteins, i.e. approximately 2% of total proteome (Martyniuk and Denslow, 2009).

Last decade efforts have been directed to develop alternative, non-gel based methods such as isobaric tagging for relative and absolute quantification (iTRQ®) (Ross et al., 2004) or isotope coded affinity tags (ICAT) couples to LC/MS (Schmidt et al., 2004). Nevertheless, several authors concluded that no single approach for all proteome analysis is available. Actually, proteins are physically and chemically much more diverse than nucleic acids and the number of protein species exceeds several folds the number of genes; both characteristics hinder the quantitative analysis of complex samples (Canovas et al., 2004) and make all current analytical technologies important and complementary (Lopez, 2007; Martyniuk and Denslow, 2009).

5.3.3. Metabolomics

The identification of alterations in the global metabolite profile, known as metabolomics, is the ultimate level of post-genomic analysis and it has been included in the group of profiling methods proposed as valuable tools for safety assessment (FAO/WHO, 2002). Estimations of the number of chemical compounds present in the plant kingdom, although considerably variable, seems to be in the range of 100,000 to 200,000 (Oksman-Caldentey and Inze, 2004); and they present a huge diversity. Due to this complexity, only the combination of many analytical techniques will allow a full description of the metabolome status of an organism (Roessner et al., 2001). Roessner and co-workers (Roessner et al., 2001) presented the first plant metabolomic approach; using gas chromatography (GC) coupled with mass spectrometry (MS) they simultaneously compared 88 compounds in 9 transgenic potato lines and further analysed the effects of environmental conditions on 86 metabolites in wild-type potato tubers. GC provides a high resolution compound separation and can also be coupled to flame ionisation detector (FID). Although GC-MS and GC-FID are sensitive and currently the most popular instruments for global metabolic profiling, thermolabile compounds are missed. Additionally, some metabolites are non volatile and need prior chemical derivatisation. LC-MS is an important additional technology for plants because it provides a resource to analyse large groups of secondary metabolites often present in plant tissues (Verhoeven et al., 2006). Recently, two separation techniques are also used in combination to MS, capillary electrophoresis (CE) (Sato et al., 2004; Soga et al., 2003) and Fourier transform ion-cyclotron resonance (FT-ICR) (Hirai et al., 2004; Murch et al., 2004). Nuclear magnetic resonance (NMR) is an alternative to the MS based approaches for metabolomic analysis. It is a quantitative and non destructive technique that can be used for rapid profiling of large number of samples (Cellini et al., 2004), but its low sensitivity limits its application for plants (Kaddurah-Daouk et al., 2009).

Plant metabolomics has become progressively more widespread during this decade, and this is reflected by the number of publications showing the wide range of applications of this technology (see reviews (Allwood et al., 2008; Fiehn et al., 2000; Hall, 2006)). In the field of the present work, metabolomic studies using NMR have been carried out to detect possible

unintended effects in different GM wheat lines (Beale et al., 2009) and in MON810 maize (Manetti et al., 2006; Piccioni et al., 2009). Using an MS- based approach, possible unintended effects of GM potato cultivars (Catchpole et al., 2005; Roessner et al., 2001) and transgenic rice (Zhou et al., 2009) were avaluated. Kristensen and collegues (Kristensen et al., 2005) used metabolomics to complement a transcriptomic approach to assess transgene unintended effects in GM *Arabidopsis* plants. Metabolites of three different transgenic maize lines grown in experimental fields were compared with their corresponding conventional lines by CE-MS (Levandi et al., 2008).

As it has been showed in the introduction of this thesis (Table 1) a number of reports based on transcriptomic, proteomic and/or metabolomic approaches to evaluate substantial equivalence of different transgenic plants have been published during the last few years. In fact, they mostly appeared during the development of this thesis and thus, results and conclusions of these papers will be examined bellow (together with our own results) in the general discussion part.

The **main objective** of the present thesis was to evaluate possible unintended effects associated to the insertion and expression of transgenes in plants, using as example MON810 maize i.e. the only GMO event that is authorised for commercialisation and cultivation in the EU. To that end the following specific objectives were proposed:

1. To compare the transcriptomic profiles of MON810 and non-GM maize plants grown under highly controlled conditions (in vitro), considering various commercial GM and near-isogenic variety pairs;

2. To assess the significance of transcriptomic differences between MON810 and conventional maize observed in plants cultured in vitro, in maize grown under agricultural field conditions;

3. To evaluate the relative contribution of diverse factors on differential gene expression between MON810 and non-GM plants: the transgenic character, conventional breeding procedures and farming practices. Two MON810 and near-isogenic pairs and two nitrogen fertilisation treatments were used as example;

4. To complement transcriptomic results through proteomics comparison of MON810 and non-GM grains grown in agricultural fields and sampled at the maturity stage typically used for forage.

III. LACK OF REPEATABLE DIFFERENTIAL EXPRESSION PATTERNS BETWEEN MON810 AND COMPARABLE COMMERCIAL VARIETIES OF MAIZE

A. Coll, A. Nadal, M. Palaudelmàs, J. Messeguer, E. Melé, P. Puigdomènech and M. Pla. “Lack of repeatable differential expression patterns between MON810 and comparable commercial varieties of maize”. *Plant Molecular Biology*. Vol. 68, issues 1-2 : p.105-117.

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Abstract

The introduction of genetically modified organisms (GMO) in many countries follows strict regulations to assure that only products that have been safety tested in relation to human health and the environment are marketed. Thus, GMOs must be authorized before use. By complementing more targeted approaches, profiling methods can assess possible unintended effects of transformation. We used microarrays to compare the transcriptome profiles of widely commercialized maize MON810 varieties and their non-GM near-isogenic counterparts. The expression profiles of MON810 seedlings are more similar to those of their corresponding near-isogenic varieties than are the profiles of other lines produced by conventional breeding. However, differential expression of □1.7 and □0.1% of transcripts was identified in two variety pairs (AristisBt/Aristis and PR33P67/PR33P66) that had similar *cryIA(b)* mRNA levels, demonstrating that commercial varieties of the same event have different similarity levels to their near-isogenic counterparts without the transgene (note that these two pairs also show phenotypic differences). In the tissues, developmental stage and varieties analyzed, we could not identify any gene differentially expressed in all variety-pairs. However, a small set of sequences were differentially expressed in various pairs. Their relation to the transgenesis was not proven, although this is likely to be modulated by the genetic background of each variety.

Keywords: GMO (Genetically Modified Organism) - MON810 - Maize - Transcriptome - Unintended effects - Expression profile

IV. GENE EXPRESSION PROFILES OF MON810 AND COMPARABLE NON-GM MAIZE VARIETIES CULTURED IN THE FIELD ARE MORE SIMILAR THAN ARE THOSE OF CONVENTIONAL LINES

A. Coll, A. Nadal, R. Collado, G. Capellades, J. Messeguer, E. Melé, M. Palaudelmàs and M. Pla. “Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in the field are more similar than are those of conventional lines”. *Transgenic research*. Vol. 18, issue 5 : p. 801-808.

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Abstract

Maize is a major food crop and genetically modified (GM) varieties represented 24% of the global production in 2007. Authorized GM organisms have been tested for human and environmental safety. We previously used microarrays to compare the transcriptome profiles of widely used commercial MON810 versus near-isogenic varieties and reported differential expression of a small set of sequences in leaves of in vitro cultured plants of AristisBt/Aristis and PR33P67/PR33P66 (Coll et al. 2008). Here we further assessed the significance of these differential expression patterns in plants grown in a real context, i.e. in the field. Most sequences that were differentially expressed in plants cultured in vitro had the same expression values in MON810 and comparable varieties when grown in the field; and no sequence was found to be differentially regulated in the two variety pairs grown in the field. The differential expression patterns observed between in vitro and field culture were similar between MON810 and comparable varieties, with higher divergence between the two conventional varieties. This further indicates that MON810 and comparable non-GM varieties are equivalent except for the introduced character.

Keywords: GMO (Genetically Modified Organism) - MON810 - Maize - Field - Unintended effects - Expression profile

V. NATURAL VARIATION EXPLAINS MOST TRANSCRIPTOMIC CHANGES AMONG MAIZE PLANTS OF MON810 AND COMPARABLE NON-GM VARIETIES SUBJECTED TO TWO N-FERTILIZATION FARMING PRACTICES

A. Coll, A. Nadal, R. Collado, G. Capellades, M. Kubista, J. Messeguer and M. Pla. “Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices”. *Plant Molecular Biology*. Vo. 73, issue 3 : p. 349-362.

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Abstract

The introduction of genetically modified organisms (GMO) in many countries follows strict regulations to ensure that only safety-tested products are marketed. Over the last few years, targeted approaches have been complemented by profiling methods to assess possible unintended effects of transformation. Here we used a commercial (Affymertix) microarray platform (i.e. allowing assessing the expression of ~1/3 of the genes of maize) to evaluate transcriptional differences between commercial MON810 GM maize and non-transgenic crops in real agricultural conditions, in a region where about 70% of the maize grown was MON810. To consider natural variation in gene expression in relation to biotech plants we took two common MON810/non-GM variety pairs as examples, and two farming practices (conventional and low-nitrogen fertilization). MON810 and comparable non-GM varieties grown in the field have very low numbers of sequences with differential expression, and their identity differs among varieties. Furthermore, we show that the differences between a given MON810 variety and the non-GM counterpart do not appear to depend to any major extent on the assayed cultural conditions, even though these differences may slightly vary between the conditions. In our study, natural variation explained most of the variability in gene expression among the samples. Up to 37.4% was dependent upon the variety (obtained by conventional breeding) and 31.9% a result of the fertilization treatment. In contrast, the MON810 GM character had a very minor effect (9.7%) on gene expression in the analyzed varieties and conditions, even though similar *cryIA(b)* expression levels were detected in the two MON810 varieties and nitrogen treatments. This indicates that transcriptional differences of conventionally-bred varieties and under different environmental conditions should be taken into account in safety assessment studies of GM plants.

Keywords: GMO (Genetically Modified Organism) - MON810 - Maize - Nitrogen stress - Transcriptome - Unintended effects - Agricultural field - Natural variation

VI. PROTEOMIC ANALYSIS OF MON810 AND COMPARABLE NON-GM MAIZE VARIETIES GROWN IN AGRICULTURAL FIELDS

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Keywords: Genetically Modified Organism (GMO); MON810; proteome; two-dimensional gel electrophoresis (2DE); mass spectrometry (MS); unexpected effects; maize.

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Abstract: Maize is the second major agricultural commodity and around one forth is currently biotech, with significant application of the insect resistant event MON810 particularly in the European Union. Grains are the major commercialized part of the plant, both harvested at late milky-starchy grains (for forage uses) or after maturity (for food and feed purposes). We assessed possible proteomic unintended effects of the MON810 transgene using two-dimensional gel electrophoresis coupled to mass spectrometry. To keep in a realistic scenario we used plants grown in agricultural fields in a region where ~50% of maize was MON810, and analyzed grains at milky-starchy stage. In maize, differential transcripts and metabolites between GM and comparable non-GM varieties tend to be variety specific. Thus, we analyzed two variety pairs, DKC6575/Tietar and PR33P67/PR33P66 which are considered representative of Food and Agriculture Organization 700 and 600 varieties commercially grown in the region.
MON810 and non-GM milky-starchy grains had virtually identical proteomic patterns, with a very small number of spots showing fold-variations in the 1-1.8 range. They were all variety specific and had divergent identities and functions. Our results support substantial equivalence between MON810 and comparable non-GM varieties.

Proteomic analysis of MON810 and comparable non-GM maize varieties grown in agricultural fields

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ABSTRACT

Maize is the second major agricultural commodity and around one forth is currently biotech, with significant application of the insect resistant event MON810 particularly in the European Union. Grains are the major commercialized part of the plant, both harvested at late milky-starchy grains (for forage uses) or after maturity (for food and feed purposes). We assessed possible proteomic unintended effects of the MON810 transgene using two-dimensional gel electrophoresis coupled to mass spectrometry. To keep in a realistic scenario we used plants grown in agricultural fields in a region where ~50% of maize was MON810, and analyzed grains at milky-starchy stage. In maize, differential transcripts and metabolites between GM and comparable non-GM varieties tend to be variety specific. Thus, we analyzed two variety pairs, DKC6575/Tietar and PR33P67/PR33P66 which are considered representative of Food and Agriculture Organization 700 and 600 varieties commercially grown in the region.

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Key words: Genetically Modified Organism (GMO), MON810, proteome, two-dimensional gel electrophoresis (2DE), mass spectrometry (MS), unexpected effects, maize

ABBREVIATIONS

2DE: two-dimensional gel electrophoresis

CRM: certified reference material

EU: European Union

FAO: Food and Agriculture Organization

GMO: Genetically Modified Organism

MS: mass spectrometry

MSDB: mass spectrometry protein sequence data base

qPCR: real-time polymerase chain reaction

V8: vegetative eight-leaf stage

DAF: days after flowering

INTRODUCTION

History reveals that high yield crop production has been achieved by different technology developments throughout years. The technology of hybrid maize developed in the 1930s and the green revolution in the 1960s made substantial contributions to crop productivity (James 2008); more recently genetic engineering represented another important step. To ensure consumers' safety strict legislation was established in many countries that regulates marketing of Genetically Modified Organisms (GMOs). The concept of substantial equivalence is the leading principle in safety assessment of GM crops (IFBC 1990), involving targeted compositional analyses and field and animal nutrition studies (EFSA 2004). However, concerns that unintended effects might remain undetected using targeted analyses encouraged the development of unbiased profiling methods as complementary tools (EC Scientific Steering Comitee 2000).

Proteomics is a non-targeted approach that has emerged in the last years as a powerful tool to detect possible unintended effects derived from genetic manipulation of various plant species. A two-dimensional gel electrophoresis (2DE) based proteomic approach was used to compare protein profiles of transgenic *Arabidopsis* (Ren et al. 2009; Ruebelt et al. 2006), tomato (Corpillo et al. 2004), potato (Goulet et al. 2010; Khalf et al. 2010; Lehesranta et al. 2005) and maize (Barros et al. 2010; Zolla et al. 2008) lines and their non-GM counterparts. These studies revealed low percentages of proteins with significantly altered levels in transgenic and non-GM lines, and the differences in spot quantities either were part of the intended effects or fell within the range of natural variability. Transciptomics (Baudo et al. 2009; Cheng et al. 2008; Dubouzet et al. 2007; El Ouakfaoui and Miki 2005; Gregersen et al. 2005; Miki et al. 2009) and metabolomics (Baker et al. 2006; Beale et al. 2009; Catchpole et al. 2005; Kristensen et al. 2005) approaches also supported the substantial equivalence of transgenic plants and comparable non-GM lines.

With 161 million ha grown in 2008, maize is the second major crop in terms of global production (FAO 2009). It is used for food and industrial applications, feed being the main usage. Worldwide about 65 % coarse grains were used for feed in 2008 (FAO 2009). Similarly, most maize grown in Europe is commonly used as feed: above 75% total maize is harvested before maturity and the entire plant is employed for forage; while the rest is harvested at maturity and grains are used for feed and food (GMO Compass 2008). Maize is the species with the most approved events (53 in 2009) (http://www.agbios.com) and MON810 is the second event approved in most countries (21 approvals in 2009) and the only biotech crop officially planted on a commercial basis in the European Union (EU) with 94.750 ha cultured in 2009 (James 2010). MON810 maize has single copy genomic insert comprising the cauliflower mosaic virus 35S promoter and maize *hsp70* intron sequences driving the expression of a synthetic *cryIA(b)* gene encoding a delta-endotoxin that acts as potent and highly specific insecticide (van Rie et al. 1989). The inserted expression cassette has a 3' truncation partially affecting the *cryIA(b)* coding sequence and resulting in the lack of terminator (Hernández et al. 2003). Transcription of the transgene was reported to read-through 3'-past the truncation site (Rosati et al. 2008) and give rise to a variety of polyadenylated transcripts of different sizes that extend to around 1 kbp downstream the truncation site (La Paz et al. 2010). A Stop codon at position +7 downstream the *cryIA(b)* sequence indicates the transgenic protein has two additional amino acids. Several authors have used analytical profiling technologies

for safety assessment of the MON810 commercial GMO. Transcriptomics, proteomics and metabolomics analyzes showed small differential transcripts / proteins / metabolites between MON810 and non-GM samples (Coll et al. 2008; Manetti et al. 2006; Piccioni et al. 2009); and the effects of varying environmental conditions were higher than those of the transgene (Barros et al. 2010; Coll et al. 2010).

Many different elite varieties have been commercialized that contain the MON810 transgene while displaying different agronomic properties. The transgenic insert is introduced into selected local varieties by classical breeding methods (Holst-Jensen et al. 2006): the transgenic line is first crossed with the elite variety and the progeny is subjected to several cycles of backcrossings with the local elite. This implies that the MON810 transgene will be placed in very different genetic backgrounds; and that genetic differences between a transgenic and a conventional "near-isogenic" variety may not solely rely on the presence of the transgene but possibly on other portions of the genomes used for breeding. Thus, comparison of MON810 and conventional maize requires selection of a MON810 variety and careful identification of the best comparable non-GM variety. The differences between GM and non-GM maize ("unexpected effects") may not be the same when different variety pairs are compared. Only a few previous works based on transcriptomics and metabolomics took into account different MON810 and near-isogenic varieties; and remarkably they showed that transcripts and metabolites mostly show altered concentrations in MON810 and non-GM samples in a variety specific manner (Coll et al. 2008; Coll et al. 2010; Levandi et al. 2008). Although this approach has been recommended by several authors (Ruebelt et al. 2006; Van Dijk et al. 2009), a proteomics based research to cover this aspect is lacking. It would be most desirable to know to which extend protein differences found in particular MON810 and non-GM pair are common to different pairs and thus, can be associated to the transgenic character of MON810, in particular in the tissues and developmental stages used for feed and food purposes.

The aim of the present study was to assess possible unintended effects of the MON810 transgene in different maize commercial varieties by means of a 2DE and mass spectrometry (MS) based proteomics approach. We analyzed representative MON810 and comparable non-GM variety pairs at the most relevant grain maturity stage from the economic perspective: late milky-starchy grains (used in forages). Samples were collected from agricultural fields.

MATERIAL & METHODS

Plant Material

Seeds from two MON810 varieties (company, date of authorization in the BOE Spanish official publication): PR33P67 (Pioneer Hi-Bred, 2003) and DKC6575 (DeKalb, Monsanto Agricultura, 2003), and their corresponding near-isogenic varieties (PR33P66 and Tietar) from the same companies were used.

Genomic DNA from 0.2 g of seeds of the two GMO varieties was isolated using the Nucleospin food kit (Macherey-Nagel Int, Easton, PA) and analyzed to confirm they were MON810. To that end they

were subjected to event specific real-time polymerase chain reaction (qPCR) (Hernández et al. 2003) using *hmg* as the endogenous control (Hernández et al. 2005). Powdered certified reference material (CRM, ref#ERM-BF413A,B,D,F), purchased from Fluka (Fluka-Riedel, Geel, Belgium) was used as control.

Seeds were grown in La Tallada d'Empordà (Girona), Catalonia, Spain (42°05'N, 3°E), where transgenic insect resistant (MON810) and conventional maize are commercially grown. Close to the sea and with a Mediterranean climate, the soil type in this area is Xerofluvent oxiaqüic, coarse-loamy, mixed, calcareous, thermic. The field under study was divided into micro-plots, 4 rows wide (row spacing 0.75 m) and 20 m long. They were sown at a density of 80,000 plants/ha (4 April 2006) and were treated following standard agricultural practices in the region. One hundred kg N/ha, 100 kg P/ha and 100 kg K/ha were applied before sowing and an additional 150 kg N/ha were side-dressed at the V8 (vegetative eight-leaf) stage. Weeds were controlled with pre-emergence application of 5 l/ha of Trophy Super (Dow Agrosciences, Indianapolis, IN, USA) (35% acetochlor + 15% atrazine + 5.8% Diclormid) and with post-emergence application of 1.25 l/ha of Samson (Syngenta, Basel, Switzerland) (4% nicosulfuron). When necessary, the fields under study were irrigated following conventional agricultural practices. Maize grains were harvested at 40 days after flowering (daf) and they were carefully checked for the absence of corn-borer, other infections and lesions. Grains of 3 plants from a single micro-plot were harvested at the same time of the day, immediately frozen in liquid nitrogen and stored at -80ºC. Two biological replicates from different micro-plots were sampled per maize variety.

Protein Extraction

Protein extraction was performed using a modified thiourea/urea protein solubilization method described by Natarajan et al. (2005). Frozen maize grains were ground in liquid nitrogen in a mortar. Approximately 100 mg of this fine powder was solubilized at room temperature in 800 μl of lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 0.1% Triton-X and 14 mM DTT) containing protease inhibitors (1 mM PMSF and 10 mM A, P, L, E-64), 1 μl/ml DNase I and 1 μl/ml RNase. Protein extracts were clarified twice at 13000rpm for 10min at 4ºC and finally ultracentrifuged at 240,000 rpm for 40 min at 4ºC. Obtained supernatants were saved and protein concentration was determined by the Bradford method (Bradford 1976).

Two-Dimensional Electrophoresis

To explore variations in protein levels associated with biological material, two replicate samples from each protein extract were firstly analyzed by using small IPG strips (7 cm, pH 4-7) (GE Healthcare, Uppsala, Sweden) in the first dimension. Protocol was similar to that used for 18cm strips (see below) with some modifications. After loading 50 μg of protein in an IPGphor™ II system (GE Healthcare), rehydration (6h at room temperature) and focusing (30 V for 6.5 h, 500 V for 1 h, 1000 V for 1 h and 5000 V for 7 h) were performed. The second dimension was carried out in a miniprotean apparatus; strips were loaded onto 12% SDS-polyacrylamide gels and were run at 50 V for 5 min and 100 V for 1 h.

Once assessed that variability within samples fall in an optimal range for proteomic analysis, three protein extractions from each sample were analyzed by 2-DE using 18 cm IPG strips and 12% SDS-PAGE gel in an Ettan™ DALT Electrophoresis Bidimensional system (GE Healthcare).

For the first dimension samples containing 400 μg of total protein were diluted in thiourea/urea rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer at the same range of IPG strips and trace of bromophenol blue) containing 1.6% (v/v) DeStreak Reagen (GE Healthcare) and loaded onto IPG strips (18 cm, pH 4-7 linear). By using an Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare), strips were firstly allowed to rehydrate for 10h at 50V and immediately they were focused with a linear voltage ramp up to 8000 V in 8 h followed by a constant voltage of 8000 V until reaching 60 kV h. For the second dimension, strips were equilibrated first for 15 min in slow agitation with a buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 10 mg/ml DTT and 0.002% (w/v) bromophenol blue and then for another 15 min in the same solution except for DTT which were replaced with 25 mg/ml iodoacetamide. Strips were then loaded onto 12% SDS-polyacrylamide gels (25 x 20 x 0.1 cm) and were run in an Ettan™ DALTsix Electrophoresis System (GE Healthcare) at 3 W/gel for 30 min followed by 20 W/gel for 4 h.

Gels were stained with colloidal Comassie Brilliant Blue (CBB) following the method by Neuhoff et al. (1988) with some modifications. After 2 h protein fixation in a 40% methanol and 10% acetic acid solution gels were incubated in a staining solution containing 2% phosphoric acid, 10% (w/v) ammonium sulphate and 0.1% (w/v) Serva Blue G-250 for 24 h at room temperature on an orbital rotator. Imaging of the stained proteins was performed using an ImageScanner™ (GE Healthcare) and images were acquired using the LabScan scanning application, in transmission mode at (16 bips) grey scale level, 300 dpi, zoom factor set at 1:1 (100%) and saved as TIFF (Tag Image File Format) files.

ImageMaster Platinum software v. 2.0 (GE Healthcare) was used to process and analyse scanned images of the 2DE gels. The analysis included spot detection, background subtraction, gel matching, generation of an average gel (master) and relative quantification of each spot. To identify quantitative differences between GM and non-GM maize grains proteome, Student t-test was performed. In the statistical analysis only spots present in at least three replicate gels of each sample that match with its comparator were considered. Spots with Student t p-values<0.05 and at least three fold increase or decrease in their relative quantities were further analyzed (in the absence of differential spots, the threshold was further placed at one-fold).

MS Analyses

Spots of interest were manually excised from 2DE gels and washed automatically using Multiprobe II robot (Perkin Elmer, Waltham, MA). Protein spots were firstly washed with distilled water for 15 min and incubated in 25 mM ammonium bicarbonate for 30min. They were subsequently dehydrated two times in 50% 25 mM ammonium bicarbonate, 50% acetonitrile for 30 min and tow times in 100% acetonitrile for 15 min. The gel pieces were finally dried to completion at 45ºC. Digestion was performed incubating each gel spot with 8μl of 12.5 μg/ml trypsin (Promega,

Madison, WI) in 25 mM ammonium carbonate, at 37ºC overnight. The resulting tryptic fragments were extracted in 10 μl formic acid 2% with sonication.

Tryptic peptides were analyzed by an ESI-Ion Trap mass spectrometer (Esquire HCT; Bruker Daltonik GmbH, Bremen, Germany), interfaced with an HPLC-Chip system (Agilent Technologies, Palo Alto, CA) at the Proteomic Plataform of INRA, Montpellier, France. A sample volume of 2 μl was loaded onto a C-18 enrichment cartridge (40 nL) with a flow rate of 4 μl/min of 0.1% (v/v) formic acid. After preconcentration and cleanup, peptides were separated in the column (HPLC-Chip C18, 5 μm, 75 μm x 43 mm, 40 nL enrichment column; Agilent Technologies, Palo Alto, CA) at a flow rate of 0.3μl/min using a gradient of 3% to 80% (v/v) acetonitrile in 15 min (0.1% [v/v] formic acid). Peptides were eluted into the High Capacity ion Trap (Esquire HCT; Bruker Daltonik GmbH, Bremen, Germany). Capillary voltage was 1.5-2 kV in the positive ion mode and was used a dry gas flow rate of 4.5 L/min with a temperature of 250°C. The first full-scan mass spectrum was measured for range 310 to 1800 m/z. The second scan was done to measure more exact M_r of the three major ions with higher resolution, and the third scan was done to measure the collision-induced MS/MS spectrum of the selected ions (range 100 to 2000 m/z).

Identification was performed by searching in the Mass Spectrometry protein sequence DataBase (MSDB) using the MASCOT software (http://www.matrixscience.com) (Taxonomy, viridiplantae). The main search parameters were: complete carbamidomethylation of cysteines, peptide mass tolerance +/-0.6 Da, fragment mass tolerance +/-0.8 Da, missed cleavages 1. For positive identification, the score of the result (-10Log(P)) had to be over the significance threshold level ($P<0.05$). Matches of MS/MS spectra against sequences in the database were also verified manually.

RESULTS

Experimental Design

In view of the importance of GM maize in the agricultural sector, the present work aims to compare protein profiles of MON810 and near-isogenic maize varieties by 2DE combined with MS. We used two highly commercialized GM varieties produced by different seed companies (PR33P67 and DKC6575) and their near isogenic counterparts (PR33P33 and Tietar, respectively). Agronomical data collected in the region of Girona, Spain from 2005 to 2008 (López et al. 2009) pointed DKC6575 and PR33P67 as the most representative varieties in the two most commonly cultured in the region Food and Agriculture Organization (FAO) maturity groups (700 and 600 respectively). Although they present some phenotypic differences, both have been recommended for their high yield production and low sensitivity to infections.

To get a realistic picture of proteomic differences among MON810 and non-GM comparable varieties we analyzed maize grains at the harvesting stage for forage applications (~75% maize cultivated): late milky-starchy stage. Grains were sampled from plants grown in field conditions in La Tallada d'Empordà, where 36.6% of cultured maize was transgenic in 2006 (DAR 2006). Fertilization and irrigation managements were in accordance with conventional agricultural

practices in the region and no phytosanitary treatments were applied. For each variety two replicate biological samples were prepared, each consisting on a pool of seeds harvested from three individual plants. Each sample was independently analyzed in experimental triplicates. Here, proteomics tools were used to demonstrate the similarity among samples.

Proteomic comparison of late milky-starchy grains grown under agricultural conditions

Two-DE protein profiles of late milky-starchy grains of two MON810 and the two corresponding non-GM comparable varieties (PR33P67 and PR33P66; DKC6575 and Tietar) were independently analyzed. For all four varieties, experimental replicates showed correlation values in the 67 to 77 % range, with loading differences below 15% (see Table S1 Supplementary information). Thus, all gels were included in further comparisons. For each variety, a virtual gel was obtained with all reproducible protein spots i.e. those that were present in at least 3 (out of 6) replicate maps of a given sample. The PR33P67 and PR33P66 virtual gels had 737 and 698 spots respectively, from which 601 were the same. DKC6575 and Tietar gels had 535 and 478 spots respectively, with 335 common spots. Common spots in each pair of varieties were around 70-80%.

We investigated quantitative protein differences between each MON810 variety and its comparable non-GM counterpart by comparison of all twelve 2DE gels of each pair (see representative examples of 2DE gels in Figure 1). To err on the safe side and avoid any artefacts to influence our results a filter was applied to specifically identify differential spots that were represented in the two virtual gels. The following thresholds were initially set: fold variation ≥3 and Student t p-value <0.05. No differences were observed in any of the two MON810 and non-GM varieties, indicating strong proteome similarity between comparable variety pairs. We further reduced the fold variation threshold down to 1 and a very small number of differential spots were revealed (Table 1). A total of 4 spots were differentially expressed in DKC6575 and Tietar grains, which represented 1.19% of analyzed spots. They were all induced in GM plants and fold induction rates were between 1 and 1.8. Similarly, 4 spots were differentially accumulated in PR33P67 and PR33P66 (i.e. 3 up-regulated and 1 down-regulated spots). This represented 0.67% of analyzed spots. We further re-analyzed 2DE gels looking for the possible presence of spots that were present in all six gels of a given variety while consistently not detected in gels of the comparator variety. Two additional spots were identified that were present in PR33P66 but below the detection limit in PR33P67. All differential spots were subjected to LC-MS/MS analysis and their presumed identities are listed in Table 1.

Proteins were identified from their peptide mass fingerprint by searching the MSDB database; and at least one presumed identity could be assigned to 9 out of 10 spots (scores >42, $p<0.05$ were considered significant). No protein in the green plant taxonomy could be identified in spot 7. Protein identification was mostly based on homologies to known maize proteins; however in some cases it was based on homologies to described *Oryza sativa* proteins. For some putative proteins the GO annotation was available (Supplemental Information Table S2).

DISCUSSION

Maize is the second major agricultural commodity in terms of global production; according to provisional data from FAO, 819.6 million tonnes of maize were produced in 2008 (FAO 2009) and 24% of this production was biotech (James 2008). So statistics reveal a significant application of this genetic engineered crop. The insect resistant maize event MON810 is the second event that has received regulatory approval in most countries and the only biotech crop officially planted on a commercial basis in the EU with over 60,000 ha cultured in 2006, mostly (86%) in Spain. Due to the commercial importance of MON810, we investigated the variability between this event and comparable non-GM varieties by a proteomics approach carried out on the most commercially relevant tissue and developmental stage. We used plants grown in agricultural fields and according to conventional farming practices, in a region where around 50% of maize was MON810. DKC6575 and PR33P67 are the most representative FAO 700 and 600 varieties commercially grown in the region; and grains are as a rule harvested at late milky-starchy grains for forage uses.

Our 2DE coupled to MS-MS identification approach showed that proteomics patterns in late milky-starchy grains of MON810 and comparable non-GM varieties were essentially the same. We only observed a very small number of quantitative differential spots between a particular MON810 and non-GM variety pair ($\leq$1%), fold variations were minimal and none was differentially accumulated in the two variety pairs tested. In a previous transcriptomics approach (Coll et al. 2008; Coll et al. 2010), leaves of MON810 commercial varieties had similarity levels to their near-isogenic counterparts in the 0.1% to 1.5% range; and (although some sequences were regulated in various variety pairs) most genes were regulated in a variety specific manner. Similarly, seeds of MON810 varieties seem to have unexpected metabolomic variations compared to their corresponding non-GM counterparts (Manetti et al. 2006; Piccioni et al. 2009), and most seem to be depend upon the specific variety. Levandi and co-workers (Levandi et al. 2008) analyzed three MON810 lines and its corresponding non-GM counterparts: from 27 metabolites identified by a CE-TOF-MS based approach, just two compounds (i.e. L-Carnitine and stachydrine) had different concentrations in all three comparisons. We earlier described the stability of MON810 commercial varieties in terms of transgene expression and CryIA(b) protein accumulation (in similar environmental conditions) (La Paz et al. 2010), thus discounting the differences between GM varieties to be due to different levels of transgenic protein. The variety specific regulation described seems to derive from portions of conventional genomes used to obtain each transgenic commercial variety: the MON810 character was introduced into different commercial varieties through a number of backcrossings with elite conventional varieties.

Remarkably, a 2DE based proteomics approach was used here to demonstrate the lack of consistent differences between MON810 and comparable non-GM varieties. It should be noted that only a small part of the total proteomic information was used. According to the experimental conditions, the analysis concerned a defined window in terms of pI and Mw and was restricted to soluble and abundant proteins. Additionally, our approach was based on comparison of the levels of protein spots that are consistently detected in the two compared samples. Proteomics 2DE analyses are subjected to large experimental deviations and our approach is intended to avoid the identification of false regulated proteins. However, we specially looked for the possible presence of

spots that were newly produced in large amounts in transgenic plants. No spots were detected fulfilling this criteria but two additional spots were present in all six non-GM PR33P66 gels while consistently absent in PR33P67 gels.

Even though the identities of proteins differentially accumulated in a specific variety pair should not be of great importance in discussing the unintended effects of transgenic maize events, we identified those found in PR33P67 and PR33P66 or DKC6575 and Tietar late milk-starchy grains as an example. They did not clearly point towards a specific biological process, molecular function or cellular component affected (which would be especially difficult due to the small numbers of regulated proteins). Regulated in PR33P67 and PR33P66 were a putative WD-40 repeat protein of unknown function, a protein predicted to regulate translation initiation (translation initiation factor 4E-1), a predicted heat shock protein involved in the response to heat stress (16.9 kDa class I heat shock protein 3), a protein most probably belonging to the proteasome complex and an enzyme involved in glucose and fatty acid metabolism (triose-phosphate isomerase). On the other hand, regulated in DKC6575 and Tietar were Os02g0625500, a predicted adenosine kinase (producing purines from its derivatives, without *de nuovo* synthesis), and a predicted heat shock protein involved in the response to stress and protein folding. Although a heat shock protein is induced in each GM vs. its comparable non-GM variety pair, the concrete proteins are not the same. A putative sorbitol dehydrogenase involved in oxidation reduction processes was differentially accumulated in DKC6575 vs. Tietar; the same protein was previously identified in a PR33P67/PR33P66 comparison (Zolla et al. 2008). Finally, a protein matching homocysteine S-methyltransferase-4 involved in methionine biosynthetic processes was induced in DKC6575 vs. Tietar. The mRNA levels of homocysteine S-methyltransferase-1 were regulated in leaves of some MON810 vs. non-GM plantlets, including DKC6575 and Tietar (Coll et al., 2008). Transcriptomics, proteomics and metabolomics techniques produce complementary results; and although different plant tissues, environmental conditions and development stages are compared, the identification of somehow related proteins, mRNAs or metabolites should not be surprising. In accordance to our proteomics results, the transcripts regulated in leaves of MON810 and non-GM pairs had a diversity of predicted functions, with the only remark of 4 genes related to the S metabolism in Aristis Bt and Aristis but not in other pairs (Coll et al. 2008). In a NMR based metabolomic approach, conservative 1H spectra were found but higher concentrations of ethanol, citric acid, glycine-betaine, trehalose and an unidentified compound were observed in PR33P67 and the comparable non-GM variety (Piccioni et al. 2009). With a similar approach, changes related to the nitrogen metabolism were found in seeds of the MON810 La73-Bt variety versus La73 (Manetti et al. 2006). Thus, variety specific differences among MON810 and non-GM comparable varieties seem to have largely divergent identities and functions. In a broader context, profiling studies carried out using different plant species and transgenes showed as well small and largely divergent differences between GMOs and non-GM plants (Barros et al. 2010; Baudo et al. 2009; Ren et al. 2009).

Agronomical data obtained from fields in the region under study showed high incidence of corn borer mainly affecting conventional varieties; which was associated with higher levels of fungi in non-GM plants compared to MON810 maize at physiological maturity stage (Van Dijk et al. 2009). Additionally, MON810 plants had better stay-green characteristics than their corresponding non-GM varieties along various seasons in the same region when conventional agricultural practices were

followed (Lian et al. 2006; Van Dijk et al. 2009). Stay-green refers to delayed senescence and is considered a positive agronomical property since it is associated to better plant health at the later cultural stages (although also to higher grain humidity at harvest of mature grains for feed or food). Our results indicate that no clear physiological differences exist between MON810 and comparable non-GM plants at the stage of harvesting for forage uses.

Previous observations from a number of authors indicated that the unintended effects of transgenes have very little impact, particularly when compared to the large differences observed between lines produced by conventional breeding approaches (Baudo et al. 2006; Catchpole et al. 2005; Ioset et al. 2007; Lehesranta et al. 2005). Moreover, environmental factors cause more variation in the different transcript, protein and metabolite profiles than the different genotypes (Barros et al. 2010; Coll et al. 2010; Zolla et al. 2008). Our 2DE patterns obtained from different conventional maize varieties were highly different (only around 60% matching proteins), thus further statistical analysis was not envisaged. But they seem to support that the differences between two conventionally bred varieties are larger than those between a GM variety and its non-GM counterpart.

In conclusion, protein differences were observed in MON810 and non-GM agronomic field-grown grains harvested for forage purposes. They affected less than 1.2% analysed proteins and were all variety specific. Thus, they could not be directly attributed to the MON810 transgenic character.

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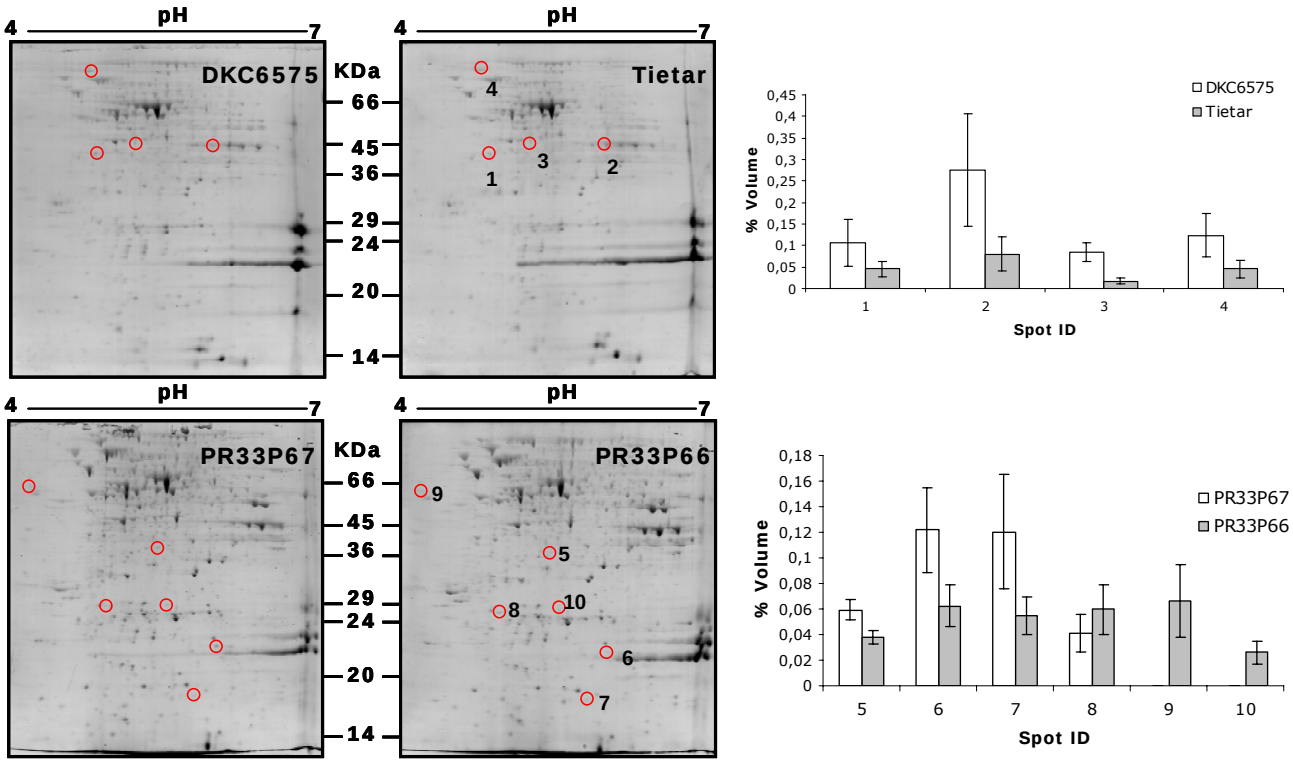
FIGURE LEGENDS

Figure 1. Protein profiles of maize grains grown under field conditions. For each sample, the image displays a representative map out of a total of 6 gels. Quantitative comparison of MON810 vs. near-isogenic maize lines [DKC6575 vs. Tietar (upper gels) and PR33P67 vs. PR33P66 (lower gels)] is shown: significantly different spots are indicated with circles and numbers according to protein identification in Table 1. Linear isoelectric focusing pH 4-7 for the first dimension and 12% SDS-PAGE gels in the second dimension were used. Gels were stained with Comassie Brillant Blue G-250. For each variety pair and differential spot, normalized spot volumes (mean and SD) are indicated in the bar graphs on the right

Figure 1


pH
4
7
DKC6575
KDa
66
45
36
29
24
20
14
Tietar
1
2
3
4
PR33P67
PR33P66
5
6
7
8
9
10
% Volume
Spot ID
DKC6575
Tietar
PR33P67
PR33P66

TABLES

Table 1. Identified spots presenting differential accumulation in late milky-starchy grains of MON810 and comparable non-GM varieties

Comparison	I/R*	Spot ID	protein**			Score	Matching peptides	Sequence coverage (%)	Mw (kDa)		pI	
			NCBI accession	UniProt accession	Protein Name				calculated	measured	calculated	measured
DKC6575/Tietar	I	1	gi:115447399	Q0DZE5_ORYSJ	Os02g0625500 (*O. Sativa*)	258	4	12	37.4	41.0	5.07	4.91
DKC6575/Tietar	I	2	gi:115477633	Q6ZBH2_ORYSJ	Os08g0545200 (*O. sativa*) (Putative sorbitol dehydrogenase)	222	5	10	40.0	45.0	6.03	6.19
DKC6575/Tietar	I	3	gi:162464417	HMT4_MAIZE	Homocysteine S-methyltransferase4 (*Z. mays*)	148	4	13	36.8	46.2	5.39	5.26
DKC6575/Tietar	I	4	gi:158513648	HSP81_ORYSI	Heat shock protein 81-1 (*O. sativa*)	582	12	16	80.4	66.0	5.00	4.87
PR33P67/PR33P66	I	5	gi:21104899	Q69X61_ORYSA	Putative WD-40 repeat protein (*O. sativa*)	170	7	17	38.1	37.2	5.43	5.43
PR33P67/PR33P66	I	6	gi:75306027	T04171	Heat shock protein (*O. sativa*)	56	3	15	16.8	19.0	6.18	5.79
PR33P67/PR33P66	I	7			unknown					22.1		5.99
PR33P67/PR33P66	R	8	gi:217974	ISZMT	Triose-phosphate isomerase (EC 5.3.1.1) (*Z. mays*)	93	3	12	27.2	27.2	5.52	4.94
PR33P67/PR33P66	R	9	gi:75220216	T51606	Probable 26S proteasome non-ATPase chain S5a (*O.sativa*)	289	6	18	42.5	59.4	4.50	4.30
PR33P67/PR33P66	R	10	gi:6016334	T01686	Translation initiation factor eIF-4F chain p26 (*Z. mays*)	50	1	5	24.7	29.0	5.52	5.50

* I:spots over-accumulated in the GM variety. R: spots under-accumulated in the GM variety
** for each spot, only the protein with the highest score is shown

Supplementary material Table S1. Two-DE technical parameters: correlation among the three experimental replicates and the two biological replicates analyzed per variety

	Plant variety			
	PR33P67	**PR33P66**	**DKC6575**	**Tietar**
Range of correlation among experimental replicates	77 - 72 %	76 - 67 %	85 - 72 %	96 - 77%
Loading difference among replicates	≤ 12.5 %	≤ 15 %	≤ 14.5 %	≤ 10.5 %
# matching spots*	737	698	535	478

* number of spots represented in at least 3 out of 6 replicates

Supplementary material Table S2. Gene Ontology (GO) mapping of identified spots with differential accumulation in late milky-starchy grains of MON810 and comparable non-GM varieties.

Comparison	Spot ID	Protein Name	GO		
			Biological process	Molecular function	Celular component
DKC6575/Tietar	1	Os02g0625500 (*O. Sativa)*	purine ribonucleoside salvage	adenosine kinase activity	
DKC6575/Tietar	2	Os08g0545200 (*O. sativa*) (Putative sorbitol dehydrogenase)	oxidation reduction	oxidoreductase activity, zinc ion binding	
DKC6575/Tietar	3	Homocysteine S-methyltransferase4 (*Z. mays*)	methionine biosynthetic process	homocysteine S-methyltransferase activity, zinc ion binding	
DKC6575/Tietar	4	Heat shock protein 81-1 (*O. sativa*)	protein folding, stress response	ATP binding, unfolded protein binding	cytoplasm
PR33P67/PR33P66	5	Putative WD-40 repeat protein (*O. sativa*)			
PR33P67/PR33P66	6	Heat shock protein (*O. sativa*)	response to heat		cytoplasm
PR33P67/PR33P66	7	unknown			
PR33P67/PR33P66	8	Triose-phosphate isomerase (EC 5.3.1.1) (*Z. mays*)	fatty acid biosynthetic process, gluconeogenesis, glycolysis	triose-phosphate isomerase activity	cytoplasm
PR33P67/PR33P66	9	Probable 26S proteasome non-ATPase chain S5a (*O.sativa*)			cytosol, proteosome complex
PR33P67/PR33P66	10	Translation initiation factor eIF-4F chain p26 (*Z. mays*)	regulation of translation	RNA binding, translation initiation factor activity	cytoplasm

Since their introduction on the market in 1996, the importance of transgenic plants has significantly raised. Not only the global transgenic area has increased, but also the countries culturing genetically modified (GM) crops and the number of new traits introduced in crops (James 2010). This global situation contrasts with the opposition existing in Europe, where consumers show a sense of mistrust in front of GM crops partly caused by the lack of consensus and practical problems to implement the current legislation.

From the first Directives that came into force in the EU (Council Directive 90/219/CEE; Council Directive 90/220/EEC) until now, legislation has evolved considerably being more strict and comprehensive in order to better cover consumer safety. Risk assessment for approval of new GM crops and derived products is approached by targeted analyses which include compositional analysis (e.g. nutrients, anti-nutrients, natutal toxins), toxicological and nutritional assessment, allergenecity tests, gene transfer risk assessment and molecular characterisation of the insert (EFSA, 2004; FAO/WHO, 2001; FAO/WHO, 2002; OECD, 1993) Consumers are especially concerned about this point (Kuiper et al., 2004), moreover several organisations indicated the limitations of this approach (e.g. biased conclusions could be raised since they will depend on the selection of the compounds to analyse) and recommended the use of profiling techniques as a complementary tool (FAO/WHO, 2000; OECD, 1996). Although profiling techniques per se could not demonstrate the food safety (since different transcriptomic, proteomic and/or metabolomic profiles are not a prove of being unsafe, neither identical patterns are not the absolute proof of safety) could allow detecting and evaluating the importance of potential unintended effects of GMOs.

The present work aimed to contribute to the existing knowledge on genetically modified organisms (GMOs) by studying possible unintended effects in the only GM crop commercially cultured in the EU, MON810 maize, by profiling methods. The study covered different aspects: two approaches (transcriptomics and proteomics); several environmental conditions (highly controlled in vitro conditions and in field conditions, both following current agronomic practices and applying low-N fertilisation conditions) and diverse tissues at different development stages (V2 and VT leaves and milky-starchy grains) which allowed an in-depth evaluation of possible unintended effects in this highly commercialised GM crop taken as an example.

Our first approach was based on transcriptomics. Numerous publications now demonstrate it is a promising tool to detect unintended effects of GMOs (Batista et al., 2008; Baudo et al., 2006; Cheng et al., 2008; El Ouakfaoui and Miki, 2005; Gregersen et al., 2005; Miki et al., 2009). As environmental conditions influence the physiology of plants, it is important that

GM and non-GM plants analysed had been grown under identical conditions to mainly identify possible changes related to the transgenic character (Kok and Kuiper, 2003; Kuiper et al., 2003). Therefore, we initially analysed leaves of in vitro cultured plantlets at the vegetative two-leaf (V2) stage grown under highly controlled conditions [this thesis (Coll et al., 2008)]. Our results indicated that unintended variation between MON810 and non-GM maize has very little impact. Similar conclusions have been reached for other species and transgenes e.g. *Arabidopsis* (Abdeen et al., 2010; El Ouakfaoui and Miki, 2005), wheat (Baudo et al., 2006; Gregersen et al., 2005), rice (Batista et al., 2008; Dubouzet et al., 2007) and soybean (Cheng et al., 2008).

Only a very recent publication is available that reports comparison of MON810 and conventional maize at the transcriptomic level. By comparing the DKC78-15B MON810 maize variety with its near-isogenic counterpart it corroborated our results (Barros et al., 2010). In addition, seed protein profiles of MON810 (variety PR33P67) and its non-GM near-isogenic variety have been compared by two-dimensional gel electrophoresis (2DE) followed by mass spectrometry (MS) (Zolla et al., 2008). Although this publication showed that the insertion of the MON810 unique transgene did not only result in expression of the CryIA(b) protein, the number of differentially regulated proteins was found to be really low. Similar results were obtained by metabolomic approaches comparing: the same MON810 variety PR33P67 and its non-GM counterpart grown in environmentally controlled growth chambers (Piccioni et al., 2009); La73-Bt and La73 plants grown under greenhouse conditions (Manetti et al., 2006); and Aristis Bt, DKC6575 and PR33P66 MON810 varieties compared with their corresponding near-isogenic varieties cultured in a field assay (Levandi et al., 2008).

In our transcriptomic study around 1.7% and 0.1% analysed sequences were differentially expressed in Aristis Bt vs. Aristis and PR33P67 vs. PR33P66, respectively. These percentages of sequences regulated between GM and non-GM plants were lower than those regulated between the two non-GM lines obtained by conventional breeding (Aristis vs. PR33P66, around 4% regulated sequences). Although this is only a measure of variation between two conventional varieties and does not represent the pool of maize genetic variation, this is in agreement with recent publications (Batista et al., 2008; Baudo et al., 2006; Cheng et al., 2008) demonstrating that transgenesis has smaller impact on the transcriptome than conventional breeding. It is known that genetic recombination is the major source of natural variation and it occurs in all crops, independently of the way they were initially obtained (by genetic engineering or via traditional breeding). Therefore, apart from the introduction of selected characteristics, unintended effects should be expected in both (Cellini et al., 2004). Actually, conventional breeding implies classical hybridisation, mutation breeding, marker assisted breeding and/or cell culture steps which are known to cause genetic alteration in the genome of the plant (Kok et al., 2008). But in the case of transgenesis the question is: Are all these small differences related to the insertion and/or expression of the transgene in MON810 plants?

Our results indicated that different MON810 and comparable non-GM variety pairs may have largely different percentages of regulated sequences. The procedure followed to obtain

commercial varieties may bring a reason to such difference. As it was pointed, commercial GM maize varieties are obtained after a series of crossings; and the precise process and lines used to obtain a given commercial GM variety are not provided by seed companies. Thus, the exact genetic distance between a GMO and the non-GM counterpart remain unknown outside the company. Consequently, the identified transcriptomic differences between MON810 and near-isogenic lines might also derive from these differences in genetic background. To assess the possible source of these differences the study was extended to the A188 line (a line with efficient regeneration properties, generally used for maize transformation) by analysing the highly differentially expressed sequences in Aristis Bt vs. Aristis and/or PR33P67 vs. PR33P66 by RT-qPCR. GM vs. non-GM regulated sequences with similar expression levels in A188 and the transgenic variety could correspond to remaining portions of the originally transformed plant genome. In Aristis Bt around 1/3 of the analysed sequences seemed to derive from the A188 line and, as expected due to the randomness of the process, they were involved in really different cell functions such as metabolism (minor carbohydrate (CHO) metabolism, glycolisis, secondary metabolism), DNA synthesis, stress and protein degradation. For PR33P67 just one regulated sequence seemed to be related to the residual genome of the originally transformed plant. Thus, Aristis Bt seemed to be more distantly related to Aristis than PR33P67 to PR33P66.

Apart from possible effects associated to the transgene, somaclonal variation caused by dedifferentiation and regeneration in tissue culture could be a possible source of unintended effects in transgenic plants. Somaclonal variation has been correlated with the stress imposed by transformation procedures (Larkin and Scowcroft, 1981; Filipecki and Malepszy, 2006). Batista and collegues (Batista et al., 2008) found that the acquisition of a desired trait (by transgenesis but also by mutagenesis) caused modifications on the expression levels of stress-related genes. Moreover, such modifications were kept for several generations although fewer transcripts were altered in each new generation. We cannot rule out that some of the differences we found between MON810 and non-GM maize could derive from somaclonal variation.

A narrow set of sequences were differentially expressed both in Aristis Bt / Aristis and PR33P67 / PR33P66. Among 16 common sequences, 13 were mapped and three were not assigned to a gene ontology term. Six sequences had a presumed function in metabolism: amino acid, minor CHO, secondary metabolism and S-assimilation; two sequences assigned in the transport category and three have presumed functions in regulation, RNA processing and gibberellins synthesis-degradation, respectively. These common regulated sequences did not group in a predefined functional gene category but it was interesting to see that four sequences seemed to be related to sulphur (S) metabolism or transport: sulphur starvation induced isoflavone reductase-like, adenosine 5'-phosphosulfate reductase 1, homocysteine S-methyltransferase- 1 and sulphate transporter.

None of the commonly regulated sequences had an expression pattern that might suggest they were a consequence of remaining portions of the A188 genome. So, direct effects of the transgene could not be dismissed. Among these sequences, three could be in silico mapped

and were located in different chromosomes (Maize Genetics and Genomics Database, www.maizegdb.org). This ruled out these transcriptional differences were uniquely due to position effects of the transgene.

On the other hand, as pleiotropic effects of the transgene were expected to be systematically present in all GM maize varieties, the 16 sequences commonly regulated in Aristis Bt vs. Aristis and PR33P67 vs, PR33P66 were further analysed in other GM/non-GM pairs (Helen Bt/Helen, Beles Sur/Sancia and DKC6575/Tietar) by RT-qPCR. Every variety pair had different levels of divergence and no sequences were consistently regulated in all analysed pairs. Actually, other authors had also reported that differences between GM and non-GM maize varieties were generally not conserved among variety pairs. For instance, carbon to nitrogen ratios were different between two out of eight analysed MON810 varieties and their near-isogenic counterparts (Griffiths et al., 2007). Similarly, different amino acid composition was observed in Aristis Bt/Aristis and PR33P67/PR33P66 but not in DKC6575/Tietar (Herrero et al., 2007).

For the same 16 sequences (regulated in Aristis Bt vs. Aristis and PR33P67 vs. PR33P66), we compared their expression levels in each GM variety and all five analysed conventional varieties by analysis of variance (ANOVA). The expression levels of regulated genes falls within the normal range in conventional maize varieties ($p>0.05$), as it was described for regulated proteins of three different transgenic *Arabidopsis* lines (Ruebelt et al., 2006a; Ruebelt et al., 2006b) and metabolomic variations of a GM potato (Catchpole et al., 2005).

All these results suggested that differences were not directly attributable to the MON810 characteristic and corroborated that different levels of divergence between GM/non-GM pairs depended on different backcrossing processes performed by every company to introduce the *cry* transgene into each commercial variety. In other words, differences between GM and comparable non-GM varieties are not consistent in all variety pairs so, they might be a consequence of the traditional breeding process followed to obtain commercial MON810 maize varieties.

Even if the transcriptional differences found between a given MON810 and its comparable non-GM variety are limited to a single (or to a reduced number of) commercial varieties, we classified the regulated sequences in functional categories using MapMan tool (Usadel et al., 2009). In the case of Aristis Bt vs Aristis, 245 out of 274 differentially expressed sequences could be annotated and the major functional groups were abiotic stress related sequences followed by genes associated to the regulation of transcription and RNA processing. As for sequences regulated in PR33P67 and PR33P66, 50 out of 65 were mapped and they had presumed functions mostly related to RNA regulation, transport, secondary metabolism, protein synthesis-degradation or biotic and abiotic stress.

Even though in vitro assays have clear advantages related to sample homogeneity, several publications support the importance to complement it by analysing plants grown under a range of environments (Kok and Kuiper, 2003; Kuiper et al., 2003). As maize is of major agricultural interest, our preliminary approach was to evaluate the significance of transcriptomic differences we previously found between in vitro cultured MON810 and their

corresponding near-isogenic varieties, in plants grown in the field. We analysed a total of 38 sequences that were differentially expressed in Aristis Bt/Aristis and/or PR33P67/PR33P66 grown under in vitro conditions [this thesis (Coll et al., 2009)]. Just a small set of them were also regulated in plants (at the same development stage) grown in agricultural fields. Particularly, just 4 out of 36 analysed sequences were differentially expressed in Aristis Bt vs. Aristis under field conditions and none of 11 anayesd sequences was regulated in PR33P67 vs. PR33P66. These results were in agreement with a previous publication reporting blueberry transcriptomic profiles along cold acclimation in two different environments: growth chambers and field conditions. Although low variation between biological replicates was found under both environments, higher numbers of regulated genes could be identified in treatments carried out at the cold-room than in field conditions. It suggests that plants could have not the very same response to cold when it is applied in the frame of very different environmental conditions; that is, some genes could be cold-induced under highly controlled conditions but at the same time might not function during cold acclimation under a real agricultural situation (Dhanaraj et al., 2007).

In contrast, up to around 60% of the 38 sequences analysed had differential expression levels in plants grown under in vitro and agricultural field conditions; and this was true for all four varieties analysed, Aristis Bt (23 out of 36 sequences analysed), PR33P67 (7 out of 11), Aristis (22 out of 36) and PR33P66 (6 out of 11). In vitro conditions are characterised by lower light intensity, higher sucrose and nitrogen and poorer gas exchanges as compared to agricultural field conditions in the region. This leads to anatomical and physiological modifications (Desjardins, 2007) and can explain the important gene expression differences between plants grown in these two environments, independently of them being transgenic or conventional.

This assay was further extended to analysing the same plants at the vegetative tasseling (VT) stage. A single one of the 38 sequences differentially expressed in vitro grown GM vs. non-GM V2 plants were also regulated in VT plants grown in agricultural fields (although the existence of other regulated sequences cannot be ruled out). However, comparison of two different defvelopmental stages (i.e. V2 and VT) in plants grown in agricultural fields showed larger differences (16 out of 36 analysed sequences were differentially expressed in Aristis Bt and 15 in Aristis, whereas the 11 sequences analysed in PR33P67 and PR33P66 were not regulated). Although it was limited to 38 sequences, this study suggested that both, growth conditions and plant development had more impact on gene expression patterns than the transgenic character. This conclusion could be summarised by clustering gene expression data [(for each GM and comparable non-GM pair, 36 selected genes, in vitro and field conditions, two development stages (agricultural field)]. Figure 4 shows the Aristis Bt/Aristis dendrogram (a similar pattern was obtained for PR33P67/PR33P66, although in this case only 11 sequences had been analysed). It reveals three main groups: in vitro cultured plantlets at V2 stage (I), plants grown in the field at VT stage (II) and plants grown in the field at V2 stage (III); so it points out the equivalence between MON810 and near-isogenic varieties and the major importance of environmental and developing factors in maize variability.

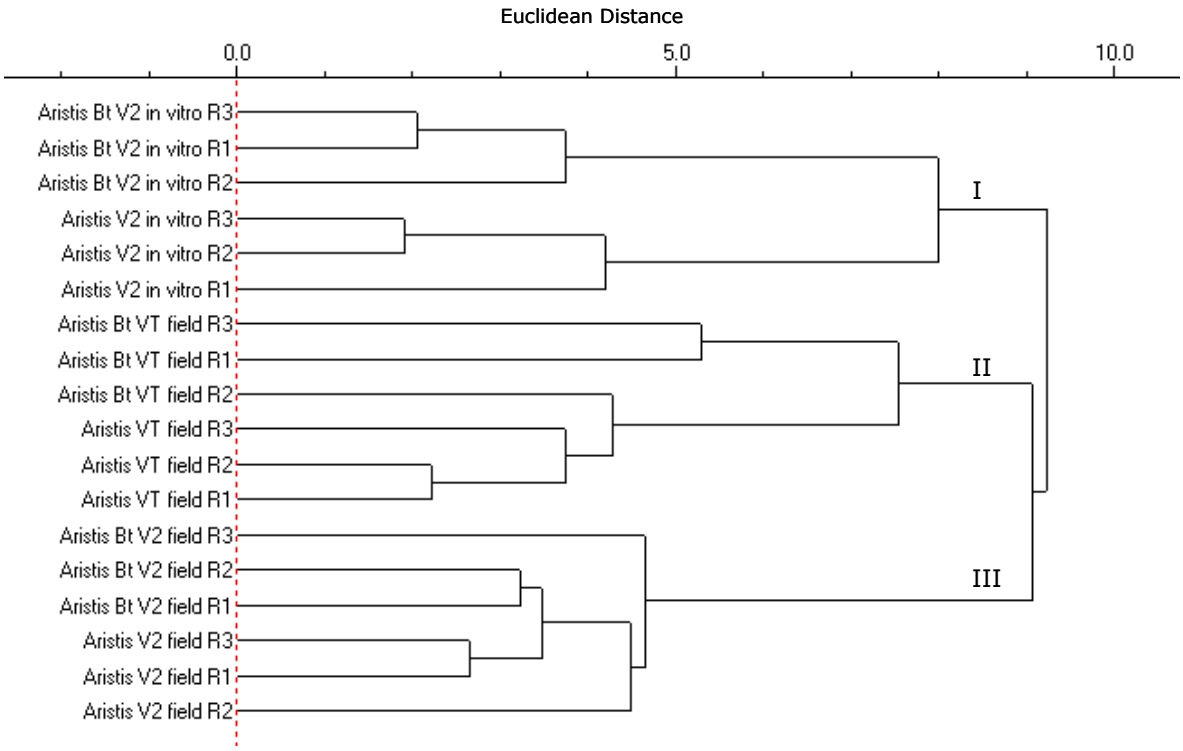


Figure 4. Hierarchical clustering of three replicates of Aristis Bt /Aristis under in vitro and field conditions at V2 and VT stage. Euclidean distances similarities between clusters were calculated using un-weighted pairs.

These first results suggested high similarity between GM and non-GM lines; however this study could not assure the existence of other differences in plants grown in agricultural fields. To assess possible unintended effects of transgenic maize under agricultural conditions we used microarray hybridisation to compare gene expression patterns of leaves of MON810 maize and its non-GM counterparts grown in agronomic fields [this thesis (Coll et al., 2010a)].

Plant genomes have a high degree of diversity; they constantly change in response to breeding processes and to environmental conditions (Parrott, 2005). Variability is especially high in field conditions due to its fluctuant characteristics. Consequently, the analysis of unintended effects of GM plants under only one selected circumstance could originate misinterpretation and false conclusions. As it has been recommended (Van Dijk et al., 2009), we took in consideration the bandwidth of natural variation analysing two highly commercialised GM/non-GM variety pairs (Helen Bt/Helen and Beles Sur/Sancia) grown in agricultural fields and considering different farming practices [two levels of nitrogen (N) fertilisation]. We choose the region of Catalunya, where 39.5% of cultured maize was MON810 in 2006 (DAR, Generalitat de Catalunya, www.gencat.cat). Plants were cultured according to the usual farming practices in the region.

Supporting our previous in vitro results [this thesis (Coll et al., 2008)], only a low number of genes were regulated between Helen Bt and Helen cultivated in the field under control and low-N conditions (0.07 and 0.13% respectively). In both treatments the identity of regulated

sequences comparing Helen Bt and Helen were similar, suggesting that transcritpomic differences were independent of the environmental conditions. However, as we expected, differences were not kept up when we extended the study to another variety pair; in fact from 14 regulated sequences between Helen Bt and Helen just half were also differentially expressed in Beles Sur and Sancia. These results highly agreed with our previous observations [this thesis (Coll et al., 2008; Coll et al., 2009)] suggesting a varietal dependence of transcriptomic differences between GM and non-GM maize.

For each particular variety, the percentage of sequences regulated in control and low-N conditions were also very low (0.17% in Helen Bt and 0.04% in Helen). But in contrast to the differences observed between GM and non-GM plants, sequences regulated by fertiliser treatments were mostly the same in Helen Bt (83%), Beles Sur (83%) and Sancia (61%). Only a small fraction of these sequences were regulated in Helen as well (13%); but this was not attributed to the transgenic character since Beles Sur behaved like the non-GM varieties and furthermore, total N contents in control and N-depleted Helen samples differed less than in the other varieties. So, differences showed in Helen were probably due to the expected deviations in experiments performed in agricultural fields. These results suggested that transgenic and near-isogenic maize lines have similar responses to the environmental conditions studied.

Evaluating the transcriptional maize response under N stress was not the main objective of this study; on the contrary fertiliser treatments were included in the assay as an example to cover different environmental circumstances on the assessment of unintended effects between GM and comparable non-GM varieties. However, as N is the most important nutrient for plant growth (Lea and Morot-Gaudry, 2009) and it is significantly relevant in economic and environmental terms (Frink et al., 1999; Sylvester-Bradley and Kindred, 2009) we took advantage of this study to contribute on the understanding of N plant response. The issue has been subjected to several studies based on transciptomics (Price et al., 2004; Scheible et al., 2004; Wang et al., 2000; Wang et al., 2003a) and proteomic approaches (Prinsi et al., 2009). They described the response of plants to short-term high-N stress (induced by re-supply of nitrate) but there was a lack of information about effects of chronic-N stress in field conditions.

In that respect Lian and colleagues (Lian et al., 2006) analysed the expression profiles of hydroponically grown rice seedlings, specifically focusing on the early stage of low-N stress (as frequently occurs in agricultural fields). No regulated sequences were identified in shoots which is different from findings in our study; probably the lack of response at early stages is due to the plant large nitrogen storage capacity in leaf vacuoles (Van der Leij et al., 1998). In contrast, 471 genes (~4.5% of analysed genes) were differentially expressed in roots. Under more realistic conditions, Bi and co-workers (Bi et al., 2007) studied *Arabidopsis* plants subjected to mild- and severe-chronic-N stresses. Although mild-chronic- N stressed plantlets had clear deficiency symptoms (reduced shoot biomass and chlorophyll level) the expression level of most genes remained the same, only 52 genes (~0.2% of analysed genes) were differentially expressed. Similarly, in our study maize plants submitted to low-N

conditions presented visible agronomic differences but just 36 sequences were regulated in Helen Bt and/or Helen.

Regarding functional classification of the regulated genes, just one was directly involved in N-metabolism (glutamine synthase 2). Studies carried out under mild-N stress at an early stage (Lian et al., 2006) or after three weeks stress (Bi et al., 2007) reached similar results: genes known to be involved in N-assimilation had a little response. On the other hand, the same authors found changes in the expression of several genes involved in carbohydrate metabolism, which provides the energy required for nitrate assimilation and amino acid biosynthesis (Kaiser et al., 2000). Our study also identified two repressed enzymes implicated in energy metabolism but in this case they were part of the Krebs cycle, a series of enzyme-catalysed chemical reactions where several catabolic pathways (carbohydrate, lipid and protein catabolism) converge to generate usable energy as NADH molecules. Two transcription factors were also repressed under low-N conditions. It is understandable that plants modify the expression of transcription factors in response to alterations in the environment. That way they can regulate gene expression networks in order to adapt their growth and development. Finally, genes involved in amino acid and protein metabolism were regulated under low-N conditions. Amino acids are the major form in which N is remobilised from leaf to grains during grain filling (Lalonde et al., 2004). Recently, Howarth and co-workers (Howarth et al., 2008) demonstrated that many genes involved in the amino acid metabolism pathway had reduced expression levels in wheat plants grown under low-N conditions and harvested at anthesis and in the next 49 days. In accordance with those results we showed that three genes involved in amino acid synthesis or degradation were regulated in maize plants under low-N conditions; and three genes related to protein synthesis and posttranslational modification were also repressed.

Taking into consideration all data obtained in this study, we used Principal Component Analysis (PCA) to evaluate the impact of the three factors introduced (transgenic character, conventional variety and N conditions) that might be involved in gene expression variability. This analysis revealed that 37.4% of the variation within data was explainable by varietal characteristics (Helen Bt and Helen predominantly differ from Beles Sur and Sancia) whereas N treatment was the second factor with higher effect on gene expression. These results supported previous transcriptomic analysis of potato tubers (Van Dijk et al., 2009) which showed major differences between two potato cultivars than between fertiliser treatments. In contrast, the transgenic character had the lowest effect on gene expression variation and it only allowed clear separation between one of the two GM/non-GM analysed pairs. In consequence, we showed that also in the field conditions gene regulation between conventional varieties and N treatments is much larger than regulation due to the MON810 transgene.

To our knowledge, this study was the first published transcriptomic approach to evaluate possible unintended effects of the transgene in one of the major biotechnological crops grown in agricultural fields and taking into account the bandwidth of natural variation (different varieties and environmental conditions). In fact, the issue of substantial

equivalence between GM and non-GM plants considering natural variation of different cultivars with a history of safe use has been covered by a few reports. Baudo and co-workers (Baudo et al., 2006) and Cheng and colleagues (Cheng et al., 2008) compared gene expression profiles of transgenic wheat and soybean, respectively, with conventionally bred cultivars grown under controlled conditions. Both reports showed that transgene effects have little impact on global genomic expression, specially compared to the large differences detected between lines produced by conventional breeding. By means of a proteomics approach, 12 transgenic *Arabidopsis* lines grown in growth chambers were compared to their parental line (Ruebelt et al., 2006b) and to 12 other *Arabidopsis* ecotype lines (Ruebelt et al., 2006a) demonstrating that the differences between GM and non-GM lines did not overshoot natural variation. Finally a metabolomics comparison of in field-grown GM and conventional potato tubers was carried out (Catchpole et al., 2005). One GM and five conventional cultivars were included in the study which concluded that the metabolite composition of transgenic potato crops was within the natural metabolite range of conventional cultivars.

But natural variation on gene expression should also be investigated in different location, climates, years of harvest and farming practices (Van Dijk et al., 2009). In this respect, gene expression changes in wild-type and transgenic in vitro cultured *Arabidopsis* plants were analysed under different abiotic stresses (e.g. salt, dehydration, cold and heat) (El Ouakfaoui and Miki, 2005). In these experiments the large number of differentially expressed genes under control and abiotic stress conditions contrasted with the little effect of T-DNA insertion. Using a proteomic approach, Zolla and collegues (Zolla et al., 2008) compared transgenic maize line PR33P67 and its isogenic variety in two subsequent generations (e.g. commercial seeds and seeds obtained by plants grown under controlled conditions) taken as different environmental conditions. They also found that the environment plays the main influence on proteomic profiles of transgenic seeds. In a more realistic scenario, transgenic rice and its comparable conventional line were grown side-by-side in experimental fields. To evaluate environmental influence, different sowing dates and field sites were studied. Gas cromatography (GC) coupled to flame ionisation detector (FID) and GC-MS analysis revealed that growing conditions and gene modification had similar influence on most analysed metabolites (Zhou et al., 2009). Very recently, Barros and co-workers (Barros et al., 2010) reported a wide study to evaluate the effects of MON810 and NK603 transgenes in maize kernels during three consecutive years and/or three different locations at the transcriptome, proteome and metabolome levels. All approaches demonstrated that environmental conditions (growing seasons and locations) had a stronger overall effect than the genetic modification.

The latter publication illustrated that transcriptomics, proteomics and metabolomics show a very little impact of the transgene; but no functional correlations were identified between genes, proteins and metabolites differentially expressed in GM and non-GM plants (Barros et al., 2010). Although a limited number of publications combined different profiling technologies to evaluate possible effects of a transgene they demonstrated the importance of the integration of these multidisciplinary approaches (Dubouzet et al., 2007; Kristensen et

al., 2005). For this reason we complemented our transcriptomic study using 2DE combined with MS to identify possible proteomic differences among MON810 and non-GM maize [this thesis (Coll et al., 2010b)]. Proteomic effects of *cry1Ab* transgene in maize had been previously reported for one single variety pair (Barros et al., 2010; Zolla et al., 2008), however our study was extended to other variety pairs in order to take into consideration the variability within conventional varieties and to be able to identify possible direct effects of the transgene.

Proteins are products of gene transcription and translation; consequently they are very suitable for the detection of changes in the genome, gene regulation or in biochemical pathways (Ruebelt et al., 2006b). Moreover they could be possible toxins, antinutrients or allergens making proteomics a direct and valuable tool for risk assessment. In this sense our study focused on the most relevant tissue for human and animal safety evaluation (i.e maize grains). We compared two highly commercialised MON810 maize varieties (PR33P67 and DKC6575) and their near-isogenic counterparts (PR33P66 and Tietar, respectively) grown in agronomical fields in the region of Catalunya previously described. To minimise environmental differences because of the differential accumulation of fungi in GM and non-GM plants (consequence of the incidence of corn borer (Serra et al., 2007)) we analysed milky-starchy grains; they accumulate fewer infestation than mature grains and are economically important since they are harvested together with the entire plant for forage uses.

By showing that the inserted *cry1Ab* transgene does not cause consistent major proteomic modifications the results achieved by this approach clearly agreed with our previous transcriptomics results [this thesis (Coll et al., 2008; Coll et al., 2010a)]. At this point we have to keep in mind that the major limitation of the proteomics technology is the narrow coverage of plant's proteins, consequently only differentially expressed proteins within the soluble and abundant proteins in a defined range of pI (4-7) and Mw (16-66 KDa) were detected. In spite of this, several proteomic studies carried out with different transgenes inserted in *Arabidopsis* (Ren et al., 2009; Ruebelt et al., 2006b), potato (Badri et al., 2009; Goulet et al., 2010; Khalf et al., 2010; Lehesranta et al., 2005) and tomato (Corpillo et al., 2004) demonstrated the usefulness of this technology and reached similar conclusions. In particular, our study revealed a small number of quantitative differential spots between a GM maize variety and its corresponding non-GM counterpart and they were regulated in a varietal specific way. Differences represented 1.19 and 0.67% of analysed spots in DKC6575/Tietar and PR33P66/PR33P67, respectively.

The identification of differential spots by MS/MS proved that not only the percentages of differentially accumulated proteins were different between variety pairs, but also their identity; solely two different heat shock proteins were induced in the two GM varieties. Although the experimental conditions were not the same, a differentially accumulated protein in DKC6575 vs. Tietar (putative sorbitol dehydrogenase) had been identified in a proteomic study analysing possible unintended effects in PR33P67 maize (Zolla et al., 2008). It had also been described as a major protein putatively responsible for variations between

Arabidopsis ecotypes (Chevalier et al., 2006). Thus, its identification in other proteomic approaches was not surprising. When we extended the comparison to transcriptomic and metabolomic approaches, homocysteine-S methyltransferase-4 was identified as a protein with higher accumulation levels in DKC6575 than Tietar maize grains, and their mRNA levels were also regulated in leaves of several MON810 vs. GM varieties [this thesis (Coll et al., 2008)]. In addition, a putative WD-40 repeat protein, a translation initiation factor, a protein probably belonging to the proteosome complex and a triose-phosphate isomerase were differentially accumulated in PR33P67 vs. PR33P66 maize grains. Finally, an adenosine kinase was overrepresented in DKC6575 maize compared to its near-isogenic line. No functional correlations could be identified between this proteomic work and previously reported meabolomic analyses of MON810 maize (Levandi et al., 2008; Manetti et al., 2006; Piccioni et al., 2009). However, all reports comparing MON810 maize varieties and their corresponding non-GM lines pointed towards the same conclusion.

Taken together, the data obtained in this thesis (along with similar studies based on transcriptomics, proteomics or metabolomics carried out by other researchers on several transgenic plants) indicate that transgenic crops could be considered substantially equivalent to their conventional counterparts because their variability fall within the range of natural variability. In spite of this, and considering random integration of the transgene in the plant genome, general conclusions can not be extracted and a case-by–case study is required for every new GM event. In fact, the European regulation forces companies to demonstrate that a novel GM crop is substantially equivalent to its traditional counterpart; contrary to the new traditionally bred plants, which are not assessed for unintended effects on a routine basis. In any type of breeding procedure unintended genetic effects may occur, actually, we found that traditional breeding introduced more variability than recombinant DNA techniques. In this sense, our results strongly support the impression about the current process of safety assessment of new plant varieties presented by Kok and collegues (Kok et al., 2008). GM plants are required to follow a separate safety evaluation process before commercialisation, which is important. However, it is significant as well to follow the developments on conventional plant breeding from the safety assessment perspective. Kok and co-workers (Kok et al., 2008) suggested screening all new plant varieties (GM and conventionally bred) by applying the comparative safety evaluation as the beginning point and, depending on the results carry out an in depth toxicological and nutritional study. Finally, we also corroborated the importance of including natural variability in the safety assessment of new plant varieties since it represents the range of consumer-acceptable variation (Hoekenga, 2008).

This thesis could help towards a more complete and unbiased information on GMOs which should facilitate decision-making by consumers, regulators, and other stakeholders.

According to the studies enclosed in this thesis, it can be **concluded**:

1. Transcriptomic microarray-based analysis of leaves of in vitro cultured plantlets at the V2 stage revealed a reduced number of differentially expressed sequences between MON810 and comparable non-GM maize. They represented 1.7% of analysed sequences in Aristis Bt and Aristis; and 0.1% in PR33P67 and PR33P66.

2. The two analysed MON810/non-GM variety pairs showed different levels of divergence in terms of number and identity of differentially expressed sequences. Moreover, the study of a number of selected genes in three other variety pairs showed that no sequence was consistently regulated in all five variety pairs. Thus, differential transcription among MON810 and near-isogenic maize could not be directly attributed to the transgene.

3. Differences between MON810 and comparable non-GM maize plants grown under highly controlled conditions were lower than those between two maize lines obtained by conventional breeding (e.g. differentially expressed sequences between Aristis and PR33P66 represented around 4% of analysed sequences) and they did not overshoot natural variation.

4. Most analysed sequences showing differential expression between in vitro cultured MON810 and near-isogenic plants were not regulated when GM and control plants were grown in agricultural fields (the same variety pairs and development stage were compared). At a later developmental stage, no analysed sequence was regulated.

5. Transcriptomic microarray based patterns of Helen Bt and Helen leaves of plants grown in agricultural fields had as low as 0.07 and 0.13 % differentially expressed sequences under conventional and low-N fertilisation, respectively. Only below 1/3rd of these sequences were regulated in Beles Sur and Sancia.

6. Chronic low-N stress in agricultural fields result in less than 0.17% differentially expressed sequences in maize leaves. They were mostly equally regulated in MON810 and non-GM varieties.

7. Transcriptional variability among MON810 and non-GM variety pairs grown in agricultural fields and subjected to two different N fertiliser treatments was mostly explained by causes other than the transgenic character. 37.4% of the variability depended on the characteristics of different varieties obtained by conventional breeding; while 31.9% depended on N fertilisation practices. The transgenic character only explained 9.7% of the observed variability. Safety assessment studies must take this into account.

8. Protein patterns of MON810 and comparable non-GM grains grown in agricultural fields and sampled at the maturity stage typically used for forage had below 1.19% differentially accumulated proteins, and they were not the same in different variety pairs.

9. From the transcriptomics and proteomics results described in this thesis, the variation added as a consequence of the MON810 transgene is small in relation to the existing variation in the commercialised gene pool; and this is one of the parameters that should be taken into account in the whole risk evaluation. Although a holistic assessment should be performed case by case, our results seem to indicate that the production of GM plants with minimal unexpected effects is possible.

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