NEW PLANT BREEDING TECHNIQUES

STATE-OF-THE-ART, POTENTIAL AND CHALLENGES

Tesis Doctoral en Biociencias y Ciencias Agroalimentarias

CLAUDIA PARISI

Directores:
Emilio Rodríguez Cerezo
José Ignacio Cubero Salmerón

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AUTOR: *CLAUDIA PARISI*

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Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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TÍTULO DE LA TESIS: Análisis de la innovación en las tecnologías de mejora vegetal

DOCTORANDA: Dª CLAUDIA PARISI

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Dª Claudia Parisi analiza en su trabajo de tesis doctoral las nuevas tecnologías utilizables en Mejora Vegetal, sus posibilidades, sus costes relativos en comparación con las metodologías clásicas y, no obstante su novedad, su repercusión en la industria. El trabajo es de la máxima actualidad dado el buen número de nuevas tecnologías puestas a punto en los últimos años, algunas para sustituir a los métodos utilizados hasta ahora para la obtención de organismos modificados genéticamente y evitar problemas sociales y legales, y otras para perfeccionarlas.

Para ello, ha partido de definir el estado actual de las tecnologías incluidas y excluidas en las Directivas de la Unión Europea junto a las nuevas técnicas basadas en la biotecnología. Examina la legislación actual al respecto, en particular la europea, el estado actual legal de los derechos de propiedad intelectual en el ámbito de la Mejora vegetal y la situación de la patentes registradas de las distintas tecnologías incluidas en el estudio mediante una amplia prospección bibliográfica. Finalmente, analiza dos casos en los que compara los costes y resultados en dos casos de desarrollo actual en sendos laboratorios en España: la mejora de la calidad del trigo para panificación y el injerto en naranjo sobre patrones enanizantes modificados genéticamente, concluyendo que aun siendo más costosas las nuevas técnicas, la calidad del producto es más predecible. Los resultados los analiza considerando las patentes necesarias y las previstas para el desarrollo comercial de los nuevos productos.

Las publicaciones acompañantes son suficiente testimonio del trabajo realizado, trabajo que creo muy bien proyectado y ejecutado.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 5 de diciembre 2012

Firma de los directores

Fdo.: _____ Fdo.: José Ignacio Cubero

TÍTULO DE LA TESIS: New Plant Breeding Techniques: State-of-the-art, potential and challenges

DOCTORANDO/A: Claudia Parisi

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Durante los tres años en IPTS (Institute for Prospective Technological Studies), Claudia Parisi ha llevado a cabo un proyecto de investigación sobre nuevas técnicas de mejora genética de plantas para la preparación de su Tesis Doctoral, bajo mi supervisión.

El estudio presenta el estado de la investigación y el desarrollo de las nuevas técnicas de mejora vegetal en la UE y en otros países; así como el nivel de adopción de las nuevas técnicas por parte de la industria y de los institutos académicos. Los datos analizados proceden de búsquedas bibliográficas en bases de datos de revistas científicas y búsquedas de patentes sobre las técnicas seleccionadas, así como de un análisis de estudios de campo realizado en la UE. Asimismo, la tesis presenta una evaluación de la viabilidad técnica de algunas de las nuevas técnicas analizadas, a través de casos-estudio, junto a una evaluación de costes y beneficios de las nuevas técnicas comparándolas con la mejora convencional. Finalmente, se presenta un análisis de las cuestiones de propiedad intelectual alrededor de las nuevas técnicas analizando el nivel de libertad de operación en un uso práctico de las mismas.

El tema de las nuevas técnicas de mejora de plantas es particularmente interesante y novedoso, tanto desde el punto de vista de la innovación científica como por las implicaciones políticas de su controvertida clasificación en el marco de la legislación sobre organismos modificados genéticamente. Por estas razones es en efecto un tópico que está despertando la atención de muchos actores en el campo de la mejora, incluyendo a investigadores, mejoradores y empresarios. De eso deriva por consecuencia una Tesis Doctoral de alto valor en campo científico.

Los resultados del estudio de Claudia en el campo de las nuevas técnicas de mejora de plantas han dado lugar a la publicación de un informe de IPTS, nombrado informe de referencia para el año 2011, y a dos publicaciones como primera autora en revistas de impacto, incluyendo a la revista de alto prestigio internacional Nature Biotechnology:

- Lusser, M., **Parisi**, C., Plan, D., Rodríguez-Cerezo, E., 2011. New plant breeding techniques. State-of-the-art and prospects for commercial development. JRC Technical Report EUR 24760 EN. European Commission. Joint Research Centre.

- Lusser, M., **Parisi**, C., Plan, D., Rodriguez-Cerezo, E., 2012. Deployment of new biotechnologies in plant breeding. Nature Biotechnology 30, 231-239.

- **Parisi**, C., Rodríguez-Cerezo, E., Thangaraj, H., 2012. Analysing patent landscapes in plant biotechnology and new plant breeding techniques. Transgenic Research, 1-15.

Mientras el informe de IPTS y el artículo en Nature Biotechnology presentan los resultados del análisis sobre estado de la investigación y el desarrollo de las nuevas técnicas de mejora vegetal, la revisión publicada por la revista Transgenic Research describe un método-guía para la búsqueda de patentes en el campo de la mejora vegetal y es fruto del aprendizaje de Claudia en estos tres años en IPTS sobre el tema de propiedad intelectual y búsqueda de patentes.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 11 de DICIEMBRE de 2012

Firma del/de los director/es

Fdo.: _____ Fdo.: _____

Eduardo Rodríguez Cerezo

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First of all, I would like to thank Emilio Rodríguez-Cerezo to give me the opportunity to work in IPTS, to introduce me to the exciting topic of New Plant Breeding Techniques, to closely supervise my work in these three years and to allow me to prepare the present PhD work. Moreover, I would like to thank José Ignacio Cubero to contribute with Emilio in the direction of my PhD and for his fundamental advice and availability.

I would like to warmly thank Francisco Barro and Antonio Martín from IAS (Instituto de Agricultura Sostenible) of Cordoba and Leandro Peña and María Angeles Forner from IVIA (Instituto Valenciano de Investigaciones Agrarias) of Valencia for the data they very kindly provided to me on the case-studies analysis and for their availability and kindness in giving me all necessary explanation and additional information.

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Finally, I would like to thank my colleagues in IPTS Pascal Tillie, Laura Riesgo, Thomas Fellmann, Mauro Vigani, Koen Dillen and Francisco Areal for their support and their valuable help and advice. Thanks also to Cristian and my family for their support in these three years.

Resumen

En la última década se han desarrollado nuevas técnicas de mejora genética de plantas (NPBTs, *New Plant Breeding Techniques*) como alternativa a los métodos de mejora convencional y a la ingeniería genética. Entre estas nuevas técnicas de mejora se incluyen técnicas de mutagénesis dirigida, técnicas que emplean transgénesis como paso intermedio de mejora para obtener plantas que no contienen genes exógenos, técnicas de transformación de plantas con secuencias de ADN procedentes de especies compatibles y técnicas de injerto en las cuales la parte injertada no contiene ninguna nueva secuencia de ADN.

El desarrollo de nuevas técnicas de mejora de plantas es relativamente nuevo y revela un elevado potencial de crecimiento. La revisión de la literatura científica y de las patentes registradas muestra el elevado número de instituciones que están involucradas en el desarrollo de estas técnicas. Mientras algunas empresas son ya conocidas en el mercado de productos transgénicos, emergen también nuevas empresas e institutos académicos. El principal motivo que impulsa la adopción de estas nuevas técnicas es su potencial para la introducción de nuevas características de interés en una variedad de cultivos. No obstante, también cabe destacar que estas instituciones se enfrentan a inconvenientes como la incertidumbre sobre la clasificación de estos cultivos en el marco de la legislación sobre organismos modificados genéticamente y los límites de libertad de operación (*Freedom to Operate*) en el campo de la propiedad intelectual.

Esta tesis presenta una revisión de publicaciones científicas y patentes sobre nuevas técnicas de mejora de plantas, teniendo en cuenta distintas variables como son las instituciones involucradas en su desarrollo tanto en la Unión Europea como a escala global, el potencial para la aplicación de estas nuevas técnicas en cultivos comerciales y las características de interés adquiridas por las nuevas variedades. Asimismo esta tesis presenta una evaluación de la viabilidad de algunas de las nuevas técnicas analizadas. Para ello se han analizado distintos casos de estudio incorporando un análisis de los costes y beneficios de las nuevas técnicas en comparación con aquellos derivados de la mejora convencional. Finalmente, se presenta un análisis de cuestiones relacionadas con la propiedad intelectual de las nuevas técnicas, no sólo identificando todas las patentes necesarias para su desarrollo sino también analizando el nivel de libertad de operación en el uso práctico de estas nuevas técnicas.

Summary

New plant breeding techniques (NPBTs) have been developed in the last decade as alternatives to conventional and transformation methods. These techniques include techniques of targeted mutagenesis, techniques in which transgenesis is used only as an intermediate breeding step and the final products are free of foreign genes, transformation with DNA sequences from cross-compatible plants and grafting techniques in which the upper part does not carry any new DNA sequence.

The field of NPBTs is young and reveals a great potential. According to scientific literature and patents, many institutions are involved in the development of NPBTs: some actors are already known in the market of transgenic products, but also new companies and academies are emerging. The main driver on the adoption of these NPBTs is their technical potential for introducing desirable traits more precisely in a variety of crops. The main constraints are the regulatory uncertainty about their classification in the scope of the GMO legislation and the limits in freedom to operate that always characterises plant biotechnology.

This thesis presents a literature and patent landscape on NPBTs, illustrating the main actors involved in their development in the EU and worldwide and their potential in terms of application in commercial crops and for traits of interest. Additionally, the technical feasibility of some NPBTs, is assessed through practical case-studies, together with an evaluation of costs and benefits compared to conventional breeding techniques. Finally, the intellectual properties issues around NPBTs are analysed, not only by identifying all patents involved but also by analysing the level of freedom to operate in a practical use of NPBTs.

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List of Abbreviations

AHAS	Acetohydroxyacid synthase
ALS	Acetolactate synthase
ARMG	Antibiotic resistance genes
BAC	Belgian Biosafety Advisory Council
bar	Phosphinothricin acetyltransferase
Bt	Bacillus thuringensis
CA	Competent Authority
CaMV	Cauliflower mosaic virus
CIMMYT	International Maize and Wheat Improvement Centre
COGEM	Netherlands Commission on Genetic Modification
CPVO	European Community Plant Variety Office
CSIC	Consejo Superior de Investigaciones Científicas
CTV	Citrus Tristeza Virus
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
dsRNA	Double stranded RNAs
EC	European Commission
ECLA	European Classification
EFSA	European Food Safety Authority
EMS	Ethyl Methane Sulfonate
EPA	Environmental Protection Agency
EPO	European Patent Office
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FISH	Fluorescent In Situ Hybridization
FTO	Freedom to Operate
GA	Gibberellins
GFP	Green Fluorescent Protein
GM	Genetically Modified
GMO	Genetically Modified Organism
GUS	Beta-glucuronidase
HMW-GS	High molecular weight subunits of wheat glutenin
IAEA	International Atomic Energy Agency
IAS	Instituto de Agricultura Sostenible
IHCP	Institute for Health and Consumer Protection
INIA	Instituto Nacional de Tecnología Agraria y Alimentaria
INPADOC	International Patent Documentation Center
IP	Intellectual Property
IPK	Inositol-1,3,4,5,6-pentakisphosphate 2-kinase
IPR	Intellectual Property Rights
IPTS	Institute for Prospective Technological Studies
ISAAA	International Service for the Acquisition of Agri-biotech Applications
IVIA	Instituto Valenciano de Investigciones Agrarias
JPO	Japanese Patent Office
JRC	Joint Research Centre
MAS	Markers-Assisted Selection
MGN	Meganuclease
NHEJ	Non-homologous end-joining

nos	Nopaline synthase
NPBT	New Plant Breeding Techniques
npt	Neomycin phosphotransferase
NTTF	New Techniques Task Force
NTWG	New Techniques Working Group
ODM	Oligonucleotide Directed Mutagenesis
OECD	Organisation for Economic Co-operation and Development
ORF	Open reading frame
PAT	Phosphinothricin phosphotransferase
PCR	Polymerase Chain Reaction
PCT	Patent Cooperation Treaty
P-DNA	Plant DNA
PEG	Polyethylene glycol
PIPRA	Public Sector Intellectual Property Resource for Agriculture
PPA	Plant Patent Act
PPT	Phosphinothricin
PVPA	Plant Variety Protection Act
qRT-PCR	Quantitative Real Time PCR
RdDM	RNA dependent DNA Methylation
RNA	Ribonucleic acid
RNAi	RNA interference
SCoFCAH	Standing Committee on the Food Chain and Animal Health
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis)
TALE	Transcription Activator-Like Effector
TALEN	Transcription Activator-Like Effector Nuclease
T-DNA	Transfer DNA
TGS	Transcriptional gene silencing
Ti	Tumor-inducing
TILLING	Targeting Induced Local Lesions in Genomes
TRIPS	Trade-Related Aspects of Intellectual Property Rights
UPOV	International Union for the Protection of new Varieties of Plants
USDA	United States Department of Agriculture
WIPO	World Intellectual Property Organization
WTO	World Trade Organization
ZF	Zinc Finger
ZFN	Zinc Finger Nuclease

Chapter 1. Technical innovation in plant breeding

1.1 Introduction

Plant breeding refers to the modification of plant characteristics based on human needs and embraces a growing number of techniques for this purpose, from basic techniques of selection to more complex molecular techniques. Plant breeding began in the Neolithic, in correspondence to the switch of human habits from nomadism to sedentarism, and consequently the discovery of plant domestication. Domestication refers to the process of selection of plants that show beneficial traits for humans and the consequent genetic change of the plant population through generations.

Since the Neolithic, and especially in the last centuries, several plant breeding techniques have been developed and improved. Plant breeders today have at disposal a very big toolbox of different techniques, through which they can reach the main objective of modern agriculture: to improve production and sustainability in order to feed the growing world population (FAO, 2009; Gates Foundation, 2012).

Since the beginning of the twentieth century fundamental new tools have been introduced to further broaden the possibilities for breeding new plant varieties. Chemical- and radiation-induced mutagenesis increases the frequency of genetic variations, and hybrid seed technology generates heterozygous plants with improved yield and disease resistance. Cell biology and tissue cultures now allows the rapid production of many uniform plants and the crossing of incompatible plants (ISAAA, 2010).

Modern biotechnology contributed broadly to a new wave of innovation dating from the 1980s. Molecular marker–assisted selection and TILLING are now widely used to map and select commercially important agricultural traits (Varshney *et al.*, 2005). Plant transformation, also known as genetic engineering, exploits recombinant DNA technology to expand the gene pool available to plant breeders. The earliest transformed crops, also called genetically modified (GM) crops, reached commercial cultivation in the mid-1990s and currently the global area sown with these varieties measures 160 million hectares (James, 2011). The application of modern biotech in the 1980s resulted in new forms of regulation and governance of certain plant breeding techniques (in particular genetic engineering) and of the release of GM crops into the environment. Various legal and regulatory approaches have been adopted worldwide.

In the past two decades, additional applications of biotechnology in plants have emerged. These include techniques of targeted mutagenesis, techniques in which transgenesis is used only as an

intermediate breeding step and the final products are free of foreign genes, transformation with DNA sequences from cross-compatible plants and grafting techniques in which the upper part does not carry any new DNA sequence. Regulators, advisory bodies and scholars have recently turned their attention to the legal classification of new plant breeding techniques (COGEM, 2006b; BAC, 2007; Schouten and Jacobsen, 2008; Breyer *et al.*, 2009; Schaart and Visser, 2009). The main question addressed by these experts is whether the new techniques differ from existing ones and how the resulting products should be classified for regulatory purposes according to current definitions of genetic modification

This thesis aims to analyse potential and challenges of new plant breeding techniques. The potential of new techniques to produce innovative crop varieties will likely be affected by the regulatory framework. Therefore, the uncertainty about the regulatory status of new techniques is extensively analysed in this context. While chapter 2 will describe in detail the regulatory implications of the legal classification of plant breeding techniques, this chapter is dedicated to an overview of the most important plant breeding techniques, grouped in the next paragraphs according to their regulatory status in the EU (European Union) in the framework of the Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (GMO) (EC, 2001). Generally, the term "conventional" will always refer to techniques that are outside the scope of the GMO directive, while plant transformation refers to techniques to produce transgenic plants, which are clearly within the scope of the Directive. Finally, new plant breeding techniques (NPBTs), that are the main subject of the present study, are included in a third group, since they do not yet have a clear legislative status in the EU.

1.2 Plant Breeding Techniques not considered as GM in EU legislation (Directive 2001/18/EC)

The list of plant breeding techniques reported in the following paragraphs is not exhaustive, but is meant to give an overview of the variety of tools available to the breeder and to analyse the characteristics that differentiate this group of techniques to the techniques classified as GM techniques, which will be treated in the next section. New techniques are constantly developed and improved, in this group of breeding techniques that do not make use of recombinant DNA and are excluded from EU GMO legislation.

1.2.1 Breeding by crossing

This group of techniques refer to methods of crossing compatible plants, i.e. plants that would also cross in nature without the help of the breeder. The action of the breeder is essential in the choice of varieties to be crossed. These techniques are used since the beginning of agriculture. Two fundamental processes belong to this group: cross-pollination (breeding of a plant with another plant) and self-pollination (breeding of the plant with itself) (van de Wiel *et al.*, 2010). The latter is very important for back-crossing. This is applied when we want to restore the genotype of an elite variety that has been crossed with another variety (e.g. a wild relative) that posses a characteristic of interest. In the next generation, the elite variety lost 50% of its genetic information, thus many back-crossings are needed to recover the elite genotype, while taking care of maintaining the trait of interest received from the other variety.

In order to obtain the crosses between the selected plants, breeders usually make use of techniques like flowers emasculation, isolation of female flowers and artificial application of pollen on the female flowers (van de Wiel *et al.*, 2010).

1.2.2 Overcoming barriers for crossing

Clearly not all plants are cross-compatible. Depending on plants' geographic distribution, temporal characteristics for flowering and/or genetic distance, different level of cross-incompatibility may be present. Breeders are often interested in overcoming these barriers, in order to enlarge the gene pool available and consequently increase the possibilities of combination of useful traits in the next generations.

Additionally, many plants are not self-compatible (Silva and Goring, 2001), meaning that they cannot self-breed. A potential explanation of this mechanism could be the prevention of inbreeding and the promotion of out-crossing in order to avoid the perpetration of harmful recessive traits. Interest of breeders in self-crossing is related to the possibility of obtaining homozygous characteristics and inbred lines for hybrid production. Another important function is also back-crossing, as described in the previous paragraph.

When incompatibility barriers are geographic, spatial (female and male flowers on different plants) or temporal (release of pollen before stigma is receptive), breeders can easily find a solution by artificially aiding pollination and by conserving pollen for the time needed. However, incompatibility could be physiological and have genetic grounds, like the S-locus in self-incompatible plants (Silva and Goring, 2001). Physiological incompatibility barriers are usually

divided into two groups: pre-zygotic (pre-fertilisation) barriers, related to the events between pollination and fertilisation, and post-zygotic (post-fertilisation) barriers, related to the development of the zygote after fertilisation (van de Wiel *et al.*, 2010).

Several breeding techniques with different level of sophistication can be applied to overcome physiological incompatibility barriers (van de Wiel *et al.*, 2010). *In planta* methods include, among others, the use of pollen of a third, compatible plant to favour the entrance of the incompatible pollen, the application of electricity or chemicals (e.g. plant hormones, like gibberellins) to favour pollination and the previous treatment of incompatible pollen with high temperature or irradiations. *In vitro* methods can also be applied to facilitate breeding between incompatible varieties. Some examples of those techniques are *in vitro* pollination (pollen and stigma are put in contact in a growth medium), *in vitro* culture of excised ovules removed from the ovary before pollination, *in vitro* culture of excised embryos (also called embryo rescue technique) and *in vitro* fertilisation, in which gametes (egg cell and sperm cell protoplasts) are extracted and fused in a growth medium.

1.2.3 Change of ploidy level

The ploidy level of domesticated plants can vary. In the example of wheat, the species durum wheat (*Triticum durum*) is tetraploid, i.e. carries four sets of chromosomes, while the most common bread wheat (*Triticum aestivum*) is hexaploid – six sets of chromosomes (Matsuoka, 2011).

Breeders are sometimes interested in increasing the ploidy level (polyploidisation) to obtain a better performance of the plants or to restore fertility. Chromosome doubling can be obtained through the application of chemicals that inhibit mitosis, such as colchicine (Caperta *et al.*, 2006). In other cases it might be of interest to reduce ploidy level to the half number of chromosomes (haploidisation) by in vitro culture of anthers or ovaries (since gamete cells posses half number of chromosomes compared to somatic cells). Once haploid cells are obtained, they can be subjected to duplication of chromosomes (e.g. through colchicine) in order to obtain perfectly homozygous cells, called doubled haploids (Jain *et al.*, 1996).

1.2.4 Increasing genetic variation

A part from the classic breeding by crossing, there are more sophisticated methods to increase genetic variability. This objective is fundamental in the work of a breeder, since agricultural developments require constantly that new useful trait (e.g. disease resistance) and trait combinations are available in crops. Therefore, the breeder is encouraged to experiment with different genetic combinations and modifications until a favourable new phenotype appears.

One example of this group of techniques is the introduction into a plant´s genome of a chromosome of another species in order to gain some new characteristics (Ballesteros *et al.*, 2003a; Ballesteros *et al.*, 2003b). Wild relatives are often a source of resistance traits that were lost in domestication and that the breeder might want to re-introduce, for example through this chromosome introgression. For this objective, the most complicated phase is the creation of addition lines of the recipient species with an extra chromosome or chromosome pair from the donor species. This technique will be presented in more details for wheat and barley in chapter 8 on case-studies.

Mutation breeding consists in the creation of random mutations in the plants by treating the seeds or the pollen with ionising radiations or chemical mutagens, like EMS (Ethyl Methane Sulfonate). The possible consequences of the action of these agents at genetic level include deletions, insertions, translocation, doubling of DNA sequences or part of chromosomes and also substitution of nucleotides. However, the breeder cannot control the effects a priori, but only select the resulting mutagenised plant with the most interesting phenotype. This technique was first adopted in the late 1920s and currently thousands of commercial plant varieties are derived from mutation breeding, according to a joint report of FAO (Food and Agriculture Organization) and IAEA (International Atomic Energy Agency) (Maluszynsk *et al.*, 2000).

Cell fusion (also called somatic hybridisation) is a complex technique that allows the union of two different plant cells´ genomes and cytoplasms. This is usually performed *in vitro* by first removing the cell wall of somatic cells and obtaining protoplasts and then chemically or electrically inducing the fusion of the selected cells. Fusion products are then cultivated *in vitro* to regenerate the new plants (van de Wiel *et al.*, 2010).

1.2.5 Selection of desirable traits through molecular tools

The generation of genetic variability as described in the previous paragraphs does not follow a predetermined pattern, but usually has random effects. A fundamental process in plant breeding is therefore the selection of the traits of interest, if present. This selection can be based in a phenotypic analysis of the desired characters. For this analysis for example the plants are grown in different mediums or soils in order to check their traits of tolerance and resistance to particular diseases or abiotic conditions. In modern breeding, molecular tools have been developed to facilitate this process and make it more specific. In this regard, MAS (Markers-Assisted Selection) and TILLING (Targeting Induced Local Lesions in Genomes) are nowadays fundamental breeding techniques that will be described in the next paragraphs.

MAS is a method that makes use of molecular markers for indirect selection of difficult traits, like traits that are not phenotypically evident at the seedling stage (Varshney *et al.*, 2005). Molecular markers are strings of DNA that are spread in the plant genome and can be followed in their segregation through generations. Known genes of interest are associated to markers for genetic proximity (genetic linkage): if they are close enough they segregate together and thus the presence of the marker guarantees also the presence of the gene of interest and allows the breeder to follow its segregation during breeding.

There are many types of molecular markers: the first to be developed were restriction fragment length polymorphisms (RFLPs); others include random amplification of polymorphic DNAs (RAPDs), cleaved amplified polymorphic sequence (CAPS), simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs); the most recently developed markers are single nucleotide polymorphisms (SNPs) and single feature polymorphisms (SFPs) (Varshney *et al.*, 2005).

MAS speeds up the process of conventional plant breeding, since breeders can identify the markers already in seedlings and do not need to obtain adult plants for the selection. Additionally, MAS facilitates the improvement of traits that cannot be easily selected using conventional methods (Varshney *et al.*, 2005). The techniques for marker screening vary and are constantly evolving. Depending on the markers they include gel electrophoresis, PCR until modern high-throughput genotyping techniques. Due to its advantages and to the growing knowledge about molecular markers, genes of interest and their localisation, MAS has become a routine step in breeding of most crops (ISAAA, 2010).

TILLING is a breeding technique that was developed a decade ago in plants (McCallum *et al.*, 2000) and can be applied to basically any type of organisms. The scope of TILLING is to detect

and analyse mutations on a specific known gene. In plant breeding it can be used to select plants carrying a specific mutation of interest.

The process of TILLING includes a first phase of creation of a mutated population, in which plant seeds are subject to mutagens, like chemical mutagens (usually EMS, see previous paragraph on mutation breeding) (Kurowska *et al.*, 2011). Subsequently, seed are sown and offspring are analysed to detect the mutations of interest. The method usually employs PCR for the amplification of the target gene and different methods for the detection of the specific mutation (e.g. restriction enzymes, DHPLC or sequencing) (Kurowska *et al.*, 2011). Finally, the selected mutant is analysed phenotypically.

There are many variants of TILLING technique (called e.g. iTILLING, deTILLING, etc.) (Kurowska *et al.*, 2011). A known variant is called ECO-TILLING and is employed for the discovery of polymorphisms in natural populations (Comai *et al.*, 2004). ECO-TILLING is based on the same process that TILLING but it is used to analyse naturally occurring instead of chemically induced mutations. It is also of interest for plant breeders to explore the presence of useful traits in the natural plant populations.

1.3 Plant Breeding Techniques considered as GM in EU legislation (Directive 2001/18/EC)

Plant "transformation" refers to the introduction and integration of “foreign” DNA in plant cells and the consequent regeneration of transgenic plants (Newell, 2000). The terms "foreign" and "transgenic" are employed to underline that the DNA introduced comes from a genetic source that is not cross-compatible with the plant subject of transformation. The opposite term "cisgenic" will be illustrated in section 1.4.2.

The term GMO is often used to refer to transgenic plants (also in this study), even if the expression "genetically modified" has a broader meaning that would also include mutagenised organisms, as it will be illustrated in the chapter on regulation.

The two most successful techniques of plants transformation are *Agrobacterium*-mediated transformation and direct DNA transfer through particle bombardment. They were both discovered in the early 1980s and will be treated in more details in the next paragraphs. Other techniques have also been tested for plant transformation, with less efficiency, like

electroporation, PEG-mediated transformation and microinjection, among others, and more techniques are being developed and improved (Rao *et al.*, 2009). All these transformation techniques make generally use of recombinant DNA. This term refers to the *in vitro* combination of DNA strands from different sources, as it cannot be found naturally (e.g. a gene combined with a different promoter and inserted in a plasmid of a different organism).

The next paragraphs are meant principally to explain the concept of transgenesis and to illustrate the main techniques of plant tranformation, which will also be mentioned in the section on new plant breeding techniques.

1.3.1 *Agrobacterium*-mediated transformation

The properties of the bacterium *Agrobacterium tumefaciens* as vector for plant transformation were discovered in particular by the work of the scientists Marc van Montagu, Jozef Schell and Mary-Dell Chilton between the 1970s and the 1980s (Chilton *et al.*, 1977; Schell and Van Montagu, 1977; Chilton, 1979; Joos *et al.*, 1983).

Agrobacterium is a soil bacterium that usually infects dicotyledonous plants, which constitute one of the major groups of flowering plants. In the infection process the bacterium transfers its DNA (Ti plasmid) to the plant cells causing the integration of its genes (Chilton *et al.*, 1977; Schell and Van Montagu, 1977). Through this mechanism the bacterium uses the expression machinery of the plant cells to produce its own nutrients. The effect of the infection of *Agrobacterium* is called crown gall disease and is characterised by the formation of plant tumours near the junction of the root and the stem (Chilton *et al.*, 1977).

Agrobacterium carries a small molecule of DNA located outside its chromosome, called Ti (tumour-inducing) plasmid (Schell and Van Montagu, 1977; Joos *et al.*, 1983). The plasmid contains a genetic region, called T-region that corresponds to the DNA sequence that is transferred and integrated into the plant genome (see Figure 1). Once integrated, this sequence is called T-DNA (transfer DNA) and contains the genes necessary for tumour formation, which are expressed by the plant machinery. The T-DNA is flanked by T-DNA borders at left and right side, both of 25 bp (base pairs) that are essential for T-DNA transfer. Outside the T-region, the Ti-plasmid contains a group of genes of virulence (called *vir* genes) that are expressed by *Agrobacterium* in response to the phenolic compounds produced by the plant in reaction to a wound. *Vir* genes are several (for a complete description of their individual functions see Gelvin (2003)) and the proteins expressed include a nuclease that cleaves the T-region (by recognising

the T-DNA-borders) and additional proteins that provoke transfer and integration of the T-DNA into the plant genome.

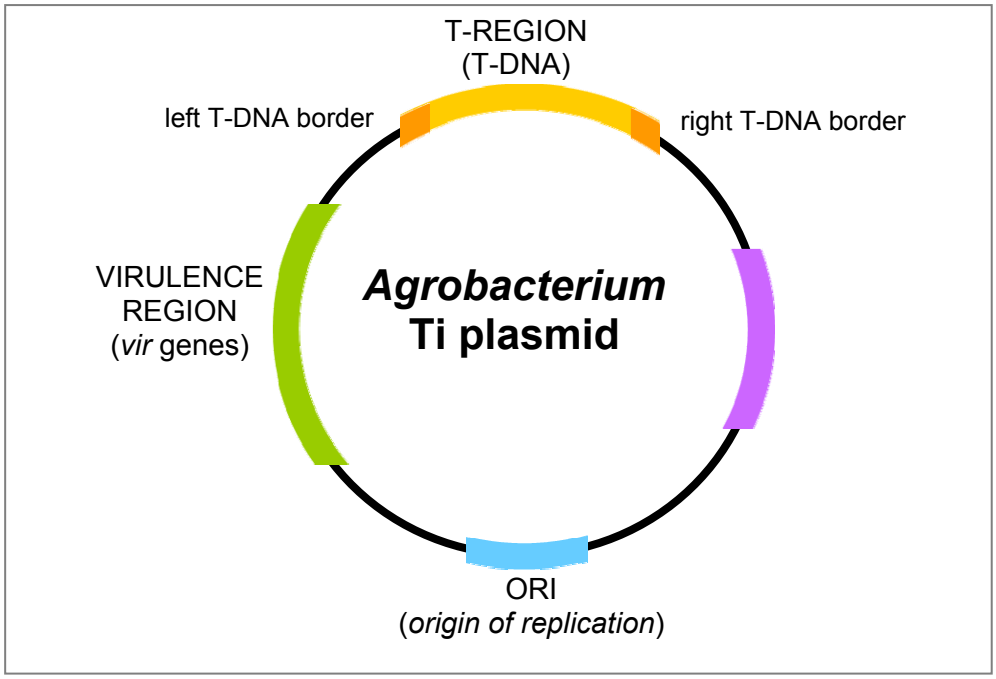

Figure 1.1 Schematic structure of the Ti plasmid of *Agrobacterium tumefaciens*

In the last decades several systems have been developed to use *Agrobacterium* as vector for gene transfer avoiding tumour formation. The common principle is the substitution of the DNA included between the T-DNA borders with the sequence to be transferred and consequently the removal of genes for tumour formation. The new sequence usually includes the gene conferring a new trait to the plant (like herbicide tolerance or pest resistance) together with selected promoters and terminators sequences and usually with marker genes for selection of transformants (see next paragraphs). *Vir* genes are maintained in the plasmid since they are necessary for the transfer of the DNA of interest into the plant cells. However, the most common strategy of transformation employs a binary vector system that includes two different smaller plasmids: a plasmid containing the T-DNA between the T-DNA borders and a selectable marker and a helper Ti plasmid containing the *vir* genes (Hoekema *et al.*, 1983).

Agrobacterium-mediated plant transformation is nowadays one the most important method for creating transgenic plants (Newell, 2000). Initially the spectrum of plants in which it was applied was limited to dicotyledonous plants only. But today also the transformation of many

monocotyledonous plants is routinely performed through *Agrobacterium*. A clear disadvantage is the low efficiency of transformation that vary among different plants and different plant tissues (Rao *et al.*, 2009).

1.3.2 Particle bombardment

Several systems of direct delivery of DNA to plant cells have been developed, like electroporation of protoplasts or plant tissue and DNA microinjection, among others (Newell, 2000). But the technique of direct DNA delivery most widely applied is the particle bombardment technique, which was discovered in the 1980s by the researcher John Sandford and his group (Klein *et al.*, 1987; Sanford, 1988) They invented the term "biolistics" (from biology and ballistics) to define the method.

In particle bombardment, the DNA sequences of interest are precipitated onto microscopic particles, usually of gold or tungsten, which are then accelerated into plant cells through a specific machine, called "gene gun" or biolistic particle delivery system. " Bombarded" plant cells can be in suspension or in tissues or plant parts (Newell, 2000). The DNA delivered into the cells is then integrated into the plant genome in one or several copies. Sometimes, truncated DNA sequences are integrated (Pawlowski and Somers, 1998).

The advantage of particle bombardment compared to *Agrobacterium* is that it does not require an interaction between two organisms (like the bacteria and the plant); therefore it is not directed to specific plant species. Another advantage is that the delivered DNA only includes the sequences that we want to have in the plant genome and no additional sequences (like the T-DNA borders of *Agrobacterium*). Another difference between particle bombardment and *Agrobacterium* is related to the number of copies delivered to each plant cells, which in biolistic methods tend to be multiple (Pawlowski and Somers, 1998). This could be a drawback or an advantage depending on the function of the delivered gene. In case in which the breeder is looking for genetic over-expression, multiple copies of the transgene could be useful.

1.3.3 Extra sequences: not just the transgene.

Independently on the method used for plant transformation, the DNA sequence integrated into the plant genome includes usually several elements: the transgene that confers a new property to the plant (main commercial traits are described in the next section), a strong promoter and a

terminator associated to the transgene, expression enhancers, and, particularly important, marker genes (also with regulatory sequences associated) to allow the selection of the transformed plants. The next paragraphs are dedicated in particular to promoters and marker genes, as they are very crucial elements in the success of plant transformation and, additionally, have very strong implications for intellectual property (see chapter 3).

Promoters

Gene promoters' function is to induce the expression of the gene associated. They can be divided into different categories: i) constitutive promoters are active constantly and in all plant tissues, ii) tissue-specific promoters are only active in specific plant tissues, iii) development-stage specific promoters only in specific periods of development and finally iv) inducible promoters are switched on and off by specific conditions, e.g. the presence of certain chemicals. All these promoters can be appropriate for transgene expression, depending on the desired trait to be introduced into the GM plant. For the most common traits in commercial transgenic plants (pest resistance and herbicide tolerance) constitutive promoters are the preferred choice, since the effect is required during the whole development period and in the whole plant, not to leave any part unprotected.

The most widely used constitutive promoter in transgenic plants is CaMV 35S, from Cauliflower mosaic virus (Odell *et al.*, 1985), which is especially efficient in dicotyledonous plants, compared to monocotyledonous. Other common promoters in transgenic plants are opines promoters, like *nos*, the promoter from the nopaline synthase. Opines are hormones produced by soil bacteria (like *Agrobacterium*) to be used as nutrients. Opines promoters are especially used for transformation of dicotyledonous plants. Another very common constitutive promoter for transformation of several organisms is the ubiquitin promoter (*Ubi*), since ubiquitines are very conserved proteins implied in several processes, like stress response. Plant ubiquitin promoters are common for plant transformation. Additional promoters commonly seen in transgenic plants are rice actin 1 promoter (Act-1) and maize alcohol dehydrogenase 1 promoter (Adh-1) (Roa-Rodríguez, 2007a).

Marker genes

Due to the generally low efficiency of the plant transformation process, there is a need for a sound method of selection of the transformed plant cells. This is usually obtained through marker genes that are co-transformed together with the gene of interest. The function of the marker gene is to confer a new property to the plant that guarantees its survival in specific

media compared to plants that do not carry it. Because of co-transformation, the presence of the marker gene implies that the selected plants also carry the gene of interest.

Marker genes usually confer to the plant the property of surviving with the presence of a toxic compound in the medium (Miki and McHugh, 2004). The most common examples are antibiotic resistance genes (called also ARMGs) and herbicide tolerance genes. They allow the transformed plants to survive media with antibiotics and herbicides, respectively. Commonly used antibiotic resistance marker genes in plant transformation are neomycin phosphotransferase II (*nptII*) that confers resistance to kanamycin, neomycin and geneticin, and hygromycin phosphotransferase (*hpt*), that confers resistance towards the antibiotic hygromycin.

Another type of selectable marker genes confers to the plants the ability to survive in the absence a specific compound (Miki and McHugh, 2004). Usual examples are genes that confer the ability of using alternative carbon sources, e.g. mannose instead of glucose with the marker gene *pmi* from the bacterium *E.Coli*. Thus the main carbon source is not present in the medium.

If the transgene confers already a selectable property, like herbicide tolerance, the transformed plants might not need any additional marker genes to be identified. A medium containing the herbicide towards which the plants are tolerant would be enough to confer a selectable advantage to the GM plants.

The widespread use of ARMGs in plant transformation has posed many questions about their safety to human and animal health, related to the possibility of transfer of resistance from the transgenic plant to the bacteria populating the gut of humans and animals (called horizontal gene transfer). The European Food Safety Authority (EFSA) was requested in 2004 to publish a scientific opinion analysing the safety assessment of ARMGs (EFSA, 2004). According to EFSA opinion, the possibility of horizontal transfer of ARMGs is negligible. However, antibiotic resistance has been devided into three categories related to the relevance of the correspondent antibiotic for human and animal health, in order to avoid the use of fundamental antibiotics for plant selection. Both *nptII* and *hpt* belong to the safest group. Nevertheless, researchers and plant breeders are constantly working on the search of alternative solutions for transformed plant selection. This is due in particular to the strong aversion of public opinion towards ARMGs. Analysed alternatives are in particular the use of herbicide tolerance genes, instead of antibiotic resistance, and techniques for an efficient removal of the marker genes, once its function in not necessary anymore (Schaart *et al.*, 2004; Terada *et al.*, 2010).

1.3.4 Current commercial applications of GM plants

Worldwide the surface cultivated with transgenic plant varieties is increasing year by year, reaching a total of 160 Million hectares in 2011 (James, 2011). The leading country in term of total hectares of GM crops is the US, followed by Brazil, Argentina, India Canada and China. Developing countries contribution to the total GM crop surface is becoming more relevant every year, reaching in 2011 50% of global GM crop. The most cultivated crops are herbicide tolerant soybean and insect resistant maize.

The traits introduced in most GM crops worldwide are still herbicide tolerance and insect resistance (James, 2011). Herbicide tolerance refers to the property of the plant to sustain the treatment with broad-spectrum herbicides, like glyphosate or glufosinate, which control most other green plants, including weeds. The most common herbicide tolerance genes used to transform plants are *cp4 epsps* (5-enolpyruvylshikimate-3-phosphate synthase) isolated from *Agrobacterium* strain CP4 and *bar* (phosphinothricin acetyltransferase) from *Streptomyces hygroscopicus* (Tan *et al.*, 2006).

Insect resistance refers to the ability of the plant to defend itself from the attack of insects, like Lepidoptera and Coleoptera. The best known group of genes used to confer insect resistance to GM crops are the Cry proteins of *Bacillus thuringensis* (Bt, from which the term "Bt crops") (Schnepf *et al.*, 1998). Cry proteins are toxic for many species of insects and are used since the 1920s as insecticides. Depending on the specific gene introduced the crop gains resistance to a group of insects. For example, the widespread cultivate Bt maize MON810 contains the gene cry1A(b) that confers resistance against European corn borer (*Ostrinia nubilalis*).

Beyond herbicide tolerance and pest resistance, other approaches are gaining increasing commercial use, in particular related to crop composition and to abiotic stress tolerance (Stein and Rodriguez-Cerezo, 2009). Examples of GM crops with changed composition are potatoes with increased starch content (like Amflora potato of BASF company), soybean and rapeseed with enhanced oil content, maize with higher lysine content and rice containing beta carotene (Golden rice). Abiotic stress tolerance refers in particular to resistance to drought and salinity.

There is additionally a growing interest in combining different traits in the same crops in order to obtain multiple characteristics in the same plant. This process of crossing different GM events is called stacking. GM plants with 2 or 3 stacked events are already commercialised (especially maize) and will be more and more common in the future (Stein and Rodriguez-Cerezo, 2009).

In the EU GM events are mostly approved for import and processing (as will be illustrated in chapter 2 on regulation). Only two GM events are currently authorised in the EU for cultivation: Bt maize MON810 in several EU countries, in particular Spain, and Amflora potato, cultivated only in Germany and Sweden in 2011.

1.4 New Plant Breeding Techniques (NPBTs) based on biotechnology

During the last decades, all plant breeding techniques described so far have been further explored and improved to give the breeders a constantly evolving toolbox. Additionally, new techniques have been developed. The subject of this study are new plant breeding techniques (NPBTs) that stem from recent advances in molecular biology and biotechnology and that are not yet clearly defined in the framework of the regulation about GMOs, as will be thoroughly illustrated in chapter 2.

The NPBTs that will be analysed in this Thesis are described in this chapter. Due to the heterogeneous nature of the techniques analysed, classification can be based on different principles. In this study they have been divided into 3 groups: i) techniques for targeted mutagenesis of plant cells, ii) techniques resulting in "null or negative segregants", i.e. those in which transgenesis is an intermediate step of the breeding process but only non transgenic plants are selected as final products, and finally iii) variants of plant transformation techniques, including the introduction of only DNA from cross-compatible species (usually known as cisgenesis-intragenesis) and commercial varieties resulting from the grafting of a non-GM scion onto a GM rootstock.

1.4.1 Targeted mutagenesis: ODM, ZFN, MGN, TALEN

Targeted mutagenesis means the obtainment of small mutations in the DNA of a plant, in pre-determined specific sites. It is also known as "site-specific mutagenesis". Targeted mutagenesis represents an important alternative to the existing techniques of mutagenesis applied to plants, illustrated in section 1.2.4, in which plant cells are exposed to chemical or physical mutagens in order to obtain random mutations in the plant DNA. The added value of targeted mutagenesis techniques is the ability to obtain only one mutation and at the desired sites, usually in order to inactivate a target gene of interest or to restore the function of a mutated gene. However,

compared to conventional mutagenesis, an essential prerequisite of targeted mutagenesis is the precise knowledge about the gene to be targeted and about the consequences of mutation.

Several targeted mutagenesis techniques developed in the last decade can be employed in plants, such as ZFN (Zinc Finger Nuclease) techniques, ODM (Oligonucleotide Directed Mutagenesis), MGN (Meganuclease) techniques and TALEN (Transcriptional Activator like Effector – Nuclease) technique. In the next paragraphs, these four techniques are described in detail. All four techniques are being used in a broad variety of organisms, playing a fundamental role their application in human therapy (Sangiuolo *et al.*, 2005; Urnov *et al.*, 2005; Lombardo *et al.*, 2007; Munoz *et al.*, 2011). Targeted mutagenesis techniques could be particularly useful in the correction of heritable point mutation that causes genetic human diseases (gene therapy). For the scope of this study, only their role in the mutagenesis of plants is considered.

Oligonucleotide directed mutagenesis (ODM)

ODM[1] is based on the use of oligonucleotides for the induction of targeted mutations in the plant genome, usually of one or a few adjacent nucleotides. The genetic changes that can be obtained using ODM include the introduction of a new mutation (replacement of one or a few base pairs), the reversal of an existing mutation or the induction of short deletions (Beetham *et al.*, 1999; Zhu *et al.*, 1999; Zhu *et al.*, 2000).

The oligonucleotides usually employed are approximately 20 to 100 nucleotides long and are chemically synthesised in order to share homology with the target sequence in the host genome, but not with the nucleotide(s) to be modified. Oligonucleotides such as chimeric oligonucleotides, consisting of mixed DNA and RNA bases, and single-stranded DNA oligonucleotides can be deployed for ODM.

Oligonucleotides can be delivered to the plant cells by methods suitable for the different cell types, including electroporation and polyethylene glycol (PEG) mediated transfection. The specific methods used for plants are usually particle bombardment of plant tissue or electroporation of protoplasts.

Oligonucleotides target the homologous sequence in the genome and create one or more mismatched base pairs corresponding to the non-complementary nucleotides. The cell's own

[1] ODM is also known as oligonucleotide-mediated gene modification, targeted gene correction, targeted gene repair, RNA-mediated DNA modification, RNA-templated DNA repair, induced targeted mutagenesis, targeted nucleotide exchange, chimeraplasty, genoplasty, oligonucleotidemediated gene editing, chimeric oligonucleotide-dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotides induced recombination, oligodeoxynucleotide-directed gene modification, therapeutic nucleic acid repair approach (the list is not exhaustive).

gene repair mechanism is believed to recognise these mismatches and induce their correction. The oligonucleotides are expected to be degraded in the cell but the induced mutations will be stably inherited.

Zinc finger nuclease (ZFN) techniques (ZFN 1,2,3)

ZFNs are proteins which have been custom-designed to cut at specific DNA sequences. They consist of a "zinc finger" domain (recognising specific DNA sequences in the genome of the plant) and a nuclease that cuts double-stranded DNA (often the nuclease FokI from *Flavobacterium okeanokoites*). The rationale for the development of ZFN techniques for plant breeding is the creation of a tool that allows the introduction of site-specific mutations in the plant genome or the site-specific integration of genes (Shukla *et al.*, 2009; Townsend *et al.*, 2009; Osakabe *et al.*, 2010; Zhang *et al.*, 2010).

The zinc finger (ZF) domain typically includes three or more individual ZF repeats that can recognize three base pairs each in a DNA sequence. Several methods for engineering ZF domains are publicly available and include: Context-dependent Assembly (CoDA), Oligomerized Pool Engineering (OPEN), and Modular Assembly (see Zinc Finger Consortium Website http://www.zincfingers.org).

As ZFNs act as heterodimers, two genes have to be delivered to the target cells, usually in an expression plasmid, with or without a short template sequence or a stretch of DNA to be inserted. Many methods are available for delivering ZFNs into plant cells, e.g. transfection, electroporation, viral vectors and *Agrobacterium*-mediated transfer.

ZFNs can be expressed transiently from a plasmid vector. Once expressed, the ZFNs generate the targeted mutation that will be stably inherited, even after the degradation of the plasmid containing the ZFNs. Alternatively, ZFN genes can be integrated into the plant genome as transgenes. In this case the offspring of the transformed plant includes plants that still carry the transgenes for the ZFNs and so have to be selected out, in order to obtain only non-transgenic plants with the desired mutation (as described in section 1.4.3 for negative segregants). The possibility of delivering ZFNs directly as proteins into plant cells is currently under investigation (Schrammeijer *et al.*, 2003; Vergunst *et al.*, 2003).

Three variants of the ZFN techniques are recognised in plant breeding (with applications ranging from producing single mutations or short deletions/insertions in the case of ZFN-1 and -2 techniques up to targeted introduction of new genes in the case of the ZFN-3 technique):

ZFN-1: Genes encoding ZFNs are delivered to plant cells without a repair template. The ZFNs bind to the plant DNA and generate site specific double-strand breaks (DSBs). The natural DNA-repair process (which occurs through non-homologous end-joining, NHEJ) leads to site-specific mutations, in one or only a few base pairs, or to short deletions or insertions (Townsend *et al.*, 2009).

ZFN-2: Genes encoding ZFNs are delivered to plant cells along with a repair template homologous to the targeted area, spanning a few kilo base pairs. The ZFNs bind to the plant DNA and generate site-specific DSBs. Natural gene repair mechanisms generate site-specific point mutations e.g. changes to one or a few base pairs through homologous recombination and the copying of the repair template (Wright *et al.*, 2005).

ZFN-3: Genes encoding ZFNs are delivered to plant cells along with a stretch of DNA which can be several kilo base pairs long and the ends of which are homologous to the DNA sequences flanking the cleavage site. As a result, the DNA stretch is inserted into the plant genome in a site-specific manner (Shukla *et al.*, 2009).

Meganuclease (MGN) techniques (MGN 1,2,3)

Meganucleases (MGNs) are very specific restriction enzymes that recognise DNA sequences of 12 to over 30 base pairs and create a double strand break (DSB) that activates repair mechanisms and DNA recombination. (Kirik *et al.*, 2000; Gao *et al.*, 2010; Kwon *et al.*, 2010). The use of MGNs for targeted mutagenesis is comparable to ZFN technique and the rationale is the same. Like for ZFN, we can distinguish three potential variants of MGN techniques: for the creation of small mutations through NHEJ (MGN 1) or through homologous recombination (MGN 2) and for the site specific insertion of a gene of interest (MGN 3).

MGNs are a very broad group of proteins expressed by several different organisms. Among them, the family group of LAGLIDAGD MGNs is commonly used for targeted mutagenesis. The DNA recognition domain of MGNs can be synthetically modified in order to direct their specificity to a broader range of target sequences. A possibility also considered in genetic engineering is the substitution of the DNA recognition domain of MGN with the recognition domain of another enzyme that possesses the target specificity of interest.

Like ZFNs, MGN act as dimers and the genes enconding the two MGNs have to be delivered to plant cells to be expressed as proteins. As for ZFNs, alternative options are currently investigated to avoid the introduction of recombinant DNA into the plant cells, like the delivery of MGNs directly as proteins.

Since the mechanism of action is comparable to ZFN technique, also for MGN three variants of the technique are recognised in plant breeding: MGN 1, similar to ZFN-1, in which only the genes encoding the MGNs are delivered to the plant cells; MGN 2, similar to ZFN 2, in which a repair template is delivered together with the genes encoding the MGNs; and MGN 3, similar to ZFN 3, in which a long stretch of DNA is delivered together with the genes encoding the MGNs and whose ends are homologous to the DNA sequences flanking the cleavage site. As for ZFN technique, the first two options are used to produce single mutations or short deletions/insertions and MGN 3 to obtain targeted introduction of new genes.

Transcription Activator-Like Effector Nuclease (TALEN) techniques

TALENs are artificial restriction enzymes that, like ZFNs, are custom-designed to cut at specific DNA sequences. TALENs are created by fusing the TAL effectors DNA binding domain to a nuclease that cuts double-stranded DNA (like nuclease FokI). TAL effectors are proteins secreted by the plant pathogen *Xanthomonas* during plant infection. TAL effectors specifically recognise and bind promoter sequences in the host plants, to activate the expression of genes affecting the disease process (Defrancesco, 2012).

TAL effectors binding domain is constituted by 17-18 repeats of around 34 aminoacids, in which aminoacids 12th and 13th of each repeat are variable and determine the recognition of a specific base pair in a DNA sequence. Therefore, sequence specificity can be modified by genetically modulating the order of the repeats (Cermak *et al.*, 2011; Morbitzer *et al.*, 2011; Weber *et al.*, 2011).

Due to similarity of action, also TALEN can be used in plants to create single mutations or short deletions/insertions or to obtain targeted introduction of new genes, like ZFN and MGN techniques (Bogdanove and Voytas, 2011; DeFrancesco, 2011; Mahfouz and Li, 2011).

1.4.2 Techniques resulting in "Negative segregants": Reverse breeding, RdDM, Early flowering

This heterogeneous group of techniques also called "transgenic construct-driven breeding techniques" (Lusser *et al.*, 2012) has as a common feature the use of transgenesis only in an intermediate step of the breeding process. The transgene used is subsequently eliminated by crossing and selection and is therefore not present in the final products, called for this reason "negative (for the absence of the transgene) segregants". This characteristic is common to the

techniques RNA-dependent DNA methylation (RdDM), Reverse Breeding and Accelerated breeding through induction of early flowering, described in the following paragraphs.

RNA-dependent DNA methylation (RdDM)

RdDM allows breeders to produce plants that do not contain foreign DNA sequences and in which no changes or mutations are made in the nucleotide sequence but in which gene expression is modified due to epigenetics.

RdDM induces the transcriptional gene silencing (TGS) of targeted genes via the methylation of promoter sequences. In order to obtain targeted RdDM, genes encoding RNAs which are homologous to promoter regions are delivered to the plant cells by suitable methods of transformation. This involves, at some stage, the production of a transgenic plant. These genes, once transcribed, give rise to double stranded RNAs (dsRNAs) which, after processing by specific enzymes, induce methylation of the target promoter sequences thereby inhibiting the transcription of the target gene (Aufsatz et al., 2002; Heilersig et al., 2006; Dalakouras et al., 2009).

In plants, methylation patterns are meiotically stable. The change in the methylation pattern of the promoter, and therefore the desired trait, will be inherited by the following generation. The progeny will include plant lines which, due to segregation in the breeding population, do not contain the inserted genes but retain the desired trait. The methylated status can continue for a number of generations following the elimination of the inserted genes (Hohn et al., 1996; Park et al., 1996). The epigenetic effect is assumed to decrease through subsequent generations and to eventually fade out, but this point needs further investigation.

Reverse Breeding

Reverse breeding is a method in which the order of events leading to the production of a hybrid plant variety is reversed. It facilitates the production of homozygous parental lines that, once hybridised, reconstitute the genetic composition of an elite heterozygous plant, without the need for back-crossing and selection (Wijnker et al., 2007; Wijnker and de Jong, 2008; Dirks et al., 2009; Wijnker et al., 2012).

The method of reverse breeding includes the following steps:

• Selection of an elite heterozygous plant that has to be reproduced;

• Suppression of meiotic recombination in the elite heterozygous plant through silencing of genes such as dmc1 and spo11 following plant transformation with transgenes encoding RNA interference (RNAi) sequences;

• Production of haploid microspores (immature pollen grains) from flowers of the resulting transgenic elite heterozygous plant;

• Use of doubled haploid (DH) technology to double the genome of the haploid microspores and to obtain homozygous cells;

• Culture of the microspores in order to obtain homozygous diploid plants;

• Selection of plant pairs (called parental lines) that do not contain the transgene and whose hybridisation would reconstitute the elite heterozygous plant.

The reverse breeding technique makes use of transgenesis to suppress meiotic recombination. In subsequent steps, only non-transgenic plants are selected. Therefore, the offspring of the selected parental lines would genotypically reproduce the elite heterozygous plant and would not carry any additional genomic change[2].

Accelerated breeding through induction of early flowering

This technique refers to the induction of early flowering in plants by transformation techniques and the subsequent use of the resulting transgenic plants in breeding. The rationale of the technique is the reduction of the time associated to each plant generation and consequently the obtainment of the final products in a shorter time. In the final breeding step, in which the early flowering trait is not necessary anymore, only non transgenic plants are selected. Therefore, the end-products are completely free of genetic modification-related DNA sequences

Several different genes linked to flowering time have been identified in plants, especially in *Arabidopsis* (Flachowsky *et al.*, 2009). The induction of early flowering can be obtained by over-expression of genes encoding transcription factor related to flowering induction (e.g. BpMADS4 from silver birch) or by silencing of genes encoding juvenility maintainance factors (e.g. TFL1 in *Arabidopsis*), in both cases a transgene would be introduced into the plants to obtain the effect (Flachowsky *et al.*, 2007a; Flachowsky *et al.*, 2007b).

[2] In addition to the producing of homozygous lines from heterozygous plants, reverse breeding offers further possible applications in plant breeding, e.g. the production of so-called chromosome substitution lines.

This technique is of particular interest in breeding of trees, in which generation time is very long and therefore the whole breeding process is very time-consuming compared to crop breeding.

1.4.3 Variants of plant transformation techniques: Cisgenesis, Intragenesis and Grafting on GM rootstock

The techniques included in this group are not new from a methodological point of view. In all of them, known plant transformation techniques are employed, usually through *Agrobacterium* or biolistic methods. The novelty of these techniques and the reason why their regulatory status is under evaluation is the way in which the final products of these techniques differ from the transgenic plants clearly considered as inside the scope of the EU GMO legislation. In the case of cisgenesis and intragenesis, the final products of transformation only carry DNA sequence from the same species or from cross-compatible ones, more similarly to products of conventional breeding, In the case of products of the form of grafting considered here, only the rootstock is transformed while the scion, and consequently plants' fruits, do not carry any foreign DNA sequence. Both techniques are described in more details in the following paragraphs.

Cisgenesis and Intragenesis

As opposed to transgenesis which can be used to insert genes from any organism, both eukaryotic and prokaryotic, into plant genomes, cisgenesis and intragenesis are terms recently created by scientists to describe the restriction of transgenesis to DNA fragments from the species itself or from a cross-compatible species. In the case of cisgenesis, the inserted genes, associated introns and regulatory elements are contiguous and unchanged. In the case of intragenesis, the inserted DNA can be a new combination of DNA fragments from the species itself or from a cross-compatible species (Nielsen, 2003; Rommens, 2004; Rommens *et al.*, 2007; Schouten and Jacobsen, 2008).

Both approaches aim to confer a new property to the modified plant. However, by definition only cisgenics could achieve results also possible by traditional breeding methods (but in a much shorter time frame). Intragenesis offers considerably more options for modifying gene expression and for trait development than cisgenesis, by allowing combinations of genes with different promoters and regulatory elements. Intragenesis can also include the use of silencing approaches, e.g. RNA interference (RNAi), by introducing inverted DNA repeats.

Cisgenic and intragenic plants are produced by the same transformation techniques as transgenic plants. The currently most investigated cisgenic plants are potato and apple, and

Agrobacterium-mediated transformation is most frequently used. However, biolistic approaches are also suitable on a case-by-case basis.

According to a very strict definition of cisgenesis and intragenesis, *Agrobacterium* T-DNA borders should not be present in the final product, even though it is technically inevitable that at least part of T-DNA borders integrates in the plant genome together with the transgene. For this reason not all scientists agree on the definition of cisgenesis and intragenesis in the matter of T-DNA borders. Contrasting safety considerations have been performing regarding cisgenesis and the risk associated to T-DNA borders (Prins and Kok, 2010; EFSA, 2012). Some scientists decided to avoid the issue by searching for sequences with equivalent functionas the T-DNA borders, but in the plant DNA, called therefore P-DNA borders (Rommens *et al.*, 2005).

Grafting non-GM varieties onto GM rootstocks

Grafting is a method whereby the above-ground vegetative component of one plant (also known as the scion), is attached to a rooted lower component (also known as the rootstock) of another plant to produce a chimeric organism with improved cultivation characteristics.

Transgenesis, cisgenesis and a range of other techniques can be used to transform the rootstock and/or scion. If a GM scion is grafted onto a non-GM rootstock, then stems, leaves, flowers, seeds and fruits will be transgenic. When a non-GM scion is grafted onto a GM rootstock, leaves, stems, flowers, seeds and fruits would not carry the genetic modification with respect to changes in genomic DNA sequences (Gal-On *et al.*, 2005; Krastanova *et al.*, 2010). For the scope of this study, only grafting of a non-GM scion onto a GM rootstock is evaluated, since in the opposite case fruits are clearly transgenic and do not pose any doubts about their classification under the current GMO legislation in the UE (see chapter 2).

Transformation of the rootstock can be obtained using traditional techniques for plant transformation, e.g. *Agrobacterium*-mediated transformation and biolistic approaches. Using genetic modification, characteristics of a rootstock including rooting capacity or resistance to soil borne diseases, can be improved, resulting in a substantial increase in the yield of harvestable components such as fruit.

If gene silencing in rootstocks is an objective this can also be obtained through RNA interference (RNAi), a system of gene silencing that employs small RNA molecules. In grafted plants, the small RNAs can also move through the graft so that the silencing signal can affect gene expression in the scion (Mallory *et al.*, 2003; Kalantidis, 2004; Tournier *et al.*, 2006). RNAi rootstocks may

therefore be used to study the effects of transmissible RNAi-mediated control of gene expression.

Chapter 2. The regulatory issues in plant breeding

2.1 Introduction

The EU has a very strict legislation on GMOs, including GM plants. According to the EU GMO legislation, a GM plant, before being released into the market, must go through an authorisation process in which the safety of the new plant for humans, animals and the environment is assessed (EC, 2001, 2003). In addition to that, strict regulations about labelling, detection and post-market monitoring are in force. All issues involve a much higher investment in terms of time and money, compared to a non GM plant variety which should only go through the variety registration process (illustrated in chapter 3) before to be put into the market.

NPBTs described previously in section 1.4 are currently being evaluated in the EU from a legislative point of view. The evaluation is being carried out by a working group established by the European Commission (EC) in October 2007 to examine these new techniques in the context of GMO legislation (EC, 2008). More details are provided in section 2.3.1. The final decision about the legislative status of NPBTs and their products (plants) will be fundamental for many companies to decide whether or not to invest in the developing and commercialisation of plant varieties produced with NPBTs.

This chapter describes in detail the regulatory requirements and implications for the release of GM plants, illustrating all the steps involved in the authorisation process in the EU. Associated costs and time will also be discussed. Additionally, the regulatory issues around NPBTs are presented.

2.2 GMO legislation

2.2.1 The EU GMO legal framework

Genetic engineering first appeared in the 1970s. The first transgenic organism was created in 1973 by Herbert Boyer and Stanley Cohen that inserted an antibiotic resistance gene into *E. coli* bacterium (Cohen and Chang, 1973). In the 1990s the EU established an extensive legal framework for the regulation of GMOs, referring to all possible organisms with the exclusion of human beings. The legal framework was amended between 2000 and 2003. The objectives of the EU GMO legislation are in particular to ensure the safety for human and animal health and the environment and the free movement of approved GMOs within the EU (Plan and Van den Eede, 2010).

Box 1 provides an overview of EU legal instruments on GMOs. The most relevant ones, and in particular in the scope of this study, are Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (EC, 2001) and Regulation (EC) No 1829/2003 on genetically modified food and feed (EC, 2003).

According to article 2 of Directive 2001/18/EC, a GMO is defined as:

"An organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination"

This definition was created in 1990 (in Council Directive 90/220/EEC, repealed by Directive 2001/18) and has remained unchanged since then. The definition focuses in particular on the technical process through which the organism has been obtained, rather than on the final product. Three lists of techniques are referred to in this definition:

- *Techniques of genetic modification*, in Annex IA, Part 1 of the Directive: they include recombinant nucleic acid techniques, micro- and macro-injection and cell fusion by means of methods that do not occur naturally

- *Techniques which are not considered to result in GMOs*, in Annex IA, Part 2: they include in vitro fertilization, natural processes like conjugation, transduction, transformation and polyploidy induction

- *Techniques of genetic modification which are excluded from the scope of the Directive*, in Annex IB: they include mutagenesis and cell fusion of plant cells which can exchange genetic material through traditional breeding methods.

Therefore, plants produced by techniques listed in Annex IA, part 1, like e.g. transgenic plants, are within the scope of Directive 2001/18 and must follow the procedure described in the Directive in order to be placed on the market. On the other side, plants produced by techniques listed in Annex IA, part 2, like e.g. crossing of certain incompatible plants through in vitro fertilisation (see section 1.2.2), and in Annex IB, like e.g. chemical or radiation-induced mutagenesis, are excluded from the scope of the Directive and therefore do not have to pass the same authorisation process.

BOX 1: Legislative instruments of EU legislation on GMOs.

Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients

Official Journal L 043 , 14/02/1997 P. 0001 - 0006

Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs

Official Journal L 109 , 06/05/2000 P. 0029 - 0042

Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration

Official Journal L 106 , 17/04/2001 P. 0001 - 0039

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance)

Official Journal L 268 , 18/10/2003 P. 0001 - 0023

Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC

Official Journal L 268 , 18/10/2003 P. 0024 - 0028

Regulation (EC) No 1946/2003 of the European Parliament and of the Council of 15 July 2003 on transboundary movements of genetically modified organisms (Text with EEA relevance)

Official Journal L 287 , 05/11/2003 P. 0001 - 0010

Commission Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms

Official Journal L 010 , 16/01/2004 P. 0005 - 0010

2004/204/EC: Commission Decision of 23 February 2004 laying down detailed arrangements for the operation of the registers for recording information on genetic modifications in GMOs, provided for in Directive 2001/18/EC of the European Parliament and of the Council (Text with EEA relevance) (notified under document number C(2004) 540)

Official Journal L 065 , 03/03/2004 P. 0020 - 0022

Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation (Text with EEA relevance)

Official Journal L 102 , 07/04/2004 P. 0014 - 0025

Commission Recommendation 2004/787/EC of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003Text with EEA relevance

Official Journal L 348 , 24/11/2004 P. 0018 - 0026

Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules

OJ L 165, 30.4.2004, p. 1–141

Regulation (EC) No 1981/2006 of the European Parliament and of the Council of 11 March 2008 amending Regulation (EC) No 1829/2003 on genetically modified food and feed, as regards the implementing powers conferred on the Commission

Official Journal L 97 , 09/04/2008 P. 0064 - 0066

Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms (Recast) (Text with EEA relevance)

Official Journal L 125 , 21/05/2009 P. 0075 - 0097

Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC (Text with EEA relevance)

Official Journal L 229 , 01/09/2009 P. 0001 - 0028

2009/770/EC: Commission Decision of 13 October 2009 establishing standard reporting formats for presenting the monitoring results of the deliberate release into the environment of genetically modified organisms, as or in products, for the purpose of placing on the market, pursuant to Directive 2001/18/EC of the European Parliament and of the Council (notified under document C(2009) 7680) (Text with EEA relevance)

Official Journal L 275 , 21/10/2009 P. 0009 – 0027

2009/866/EC: Commission Decision of 30 November 2009 authorising the placing on the market of products containing, consisting of, or produced from genetically modified maize MIR604 (SYN-IR6Ø4-5) pursuant to Regulation (EC) No 1829/2003 of the European Parliament and of the Council (notified under document C(2009) 9399) (Text with EEA relevance)

Official Journal L 314 , 01/12/2009 P. 0102 - 0105

Commission Recommendation 2010/C200/01 of 13 July 2010 on guidelines for the development of national co-existence measures to avoid the unintended presence of GMOs in conventional and organic crops

Official Journal C 200 , 22/07/2010 P. 0001 - 0005

Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired (text with EEA relevance)

Official Journal L 166 , 25/06/2011 P. 0009 – 0015

Directive 2001/18 concerns the release of GMOs into the environment, covering both experimental and commercial release. For *experimental release*, the applicant (called "notifier") must submit an application to the competent national authority of an EU country. The notification must include an evaluation of the environmental risks related to the release of the analysed GMO. The competent authority will authorise or not the experimental release of the GMO, but only limited to its Member State.

For the authorisation for *commercial release* of a GMO, the notifier must submit an application (called "notification") to the competent authority of one EU Member State. The notification must include, among other things, all detailed information about the GMO, including molecular information, environmental risk assessment, post-market monitoring plan and proposal for labelling of GM products. The competent authority prepares an opinion on the risk assessment

of the GMO. If favourable, the opinion is passed to the EC, which informs the Member States and asks for the opinion of the EFSA (European Food Safety Authority), whose role and responsibilities are described in section 2.2.2. EFSA experts carry out a complete environmental risk assessment of the new GMO and prepare a scientific opinion. The final decision is taken by The EC in collaboration with the Regulatory Committee composed of representatives of the Member States and the Council of Ministers.

While Directive 2001/18 refers to all kind of GMOs that could be released into the environment, including for instance plants, animals, insects and microorganisms, Regulation (EC) 1829/2003 is only related to food and feed. In particular it regulates the placing on the market of food or feed containing, consisting of or produced from GMOs. As an example, GM ornamental plants would be covered by the Directive 2001/18 but not by the Regulation 1829/2003. Since the core subjects of this thesis are new techniques applied to plant breeding, whose main products are of interest for food and feed industry, the main focus is on Regulation (EC) 1829/2003, which determines the authorisation process described in Figure 2.1 for the placing on the market of GM food and feed.

According to the principle "one door, one key", applications for a food or feed product containing or consisting of GMOs can be filed entirely under Regulation 1829/2003 in order to obtain an authorisation for the deliberate release of the GMO into the environment. This is the way more often chosen by applicants. Alternatively, the applicant can choose to split the application and submit it both under Directive 2001/18/EC, for the environmental risk assessment, and Regulation 1829/2003, for food and feed risk assessment.

According to Regulation (EC) No 1829/2003 (Figure 2.1), the application for approval of GM food/feed is first sent to a national competent authority. The application must include all the information about the studies performed to demonstrate that the GMO is safe for human and animal health and for the environment and the description of methods for detection, sampling and identification of the GMO. The applicant must additionally provide samples of the GM food/feed to be analysed in the laboratories for the validation of the detection method provided.

In a place of 14 days the national Competent Authority informs EFSA, which is responsible of the scientific risk assessment of the GMO regarding human and animal health and environment. EFSA must provide its scientific opinion within six months of receiving the application. If EFSA needs to request more data to the applicant in order to finalise its opinion, the time is stopped. EFSA opinion must also include the validated detection method for the GM food/feed. The method validation is carried on by the Community Reference Laboratory (CRL) of the Joint Research Centre (JRC), assisted by the European Network of GMO Laboratories (ENGL).

EFSA's scientific opinion is made available to the public and finally sent to the EC that submits a draft decision for approval to the Standing Committee on the Food Chain and Animal Health (SCoFCAH), composed of representatives of the Member States. The Committee can accept or reject the application, but only by qualified majority (two third of voters). If the Committee accepts the proposal, it is finally adopted by the EC. If the Committee rejects the proposal by qualified majority or if it does not reach the qualified majority, the proposal is passed on to the Council of Agricultural Ministers, which has a time limit of three months to reach a qualified majority for, or against, the proposal. If the Council does not act within three months or does not reach the qualified majority, then the proposal is passed back to the EC which then adopts it. The authorisation for GM food/feed is valid throughout the EU for ten years and is renewable.

In addition to the described process of authorisation for GMOs, there are other issues that differ substantially in the process of putting a GM plant variety into the market when compared with a variety obtained by non-regulated breeding techniques, as described in chapter 1. The most relevant additional requirements are the mandatory labelling of GM products, the traceability, the post-market monitoring and, once authorised and cultivated, the mandatory coexistence measures to avoid comingling with neighbouring farms. Labelling refers to the obligation of putting a label to food/feed products containing more than 0.9% of GMOs, according to Regulation 1829/2003. Traceability requirements for GMOs are defined in Regulation 1830/2003 in order to monitor all GM products in the market. Post market monitoring concerning environmental effects of GMOs is required by Regulation 1829/2003.

Finally, coexistence measure must be taken into account by GM crops farmers, in order to avoid unintended presence of GM material in other products (conventional and organic crops). The Commission Recommendation 2010/C200/01 provides guidelines for the development of national coexistence measures that can vary among EU countries, due to differences in the particular local conditions.

The functioning of the EU GMO legislative framework was subjected to an external review between 2009 and 2011, in particular on the aspects of GMO cultivation and GM food and feed. The purpose was to evaluate effectiveness and efficiency of the legislation and identify needs for adjustment. Indeed the evaluators identified some issues to be addressed that are currently considered by the European Commission. These issues in particular refer to the flexibility of the GMO legislative system, the socio-economic impact of GMOs, the reinforcement of monitoring activities and the evaluation of NPBTs. More information about the evaluation exercise is available in the EC Website through this link: http://ec.europa.eu/food/food/biotechnology/evaluation/index_en.htm.

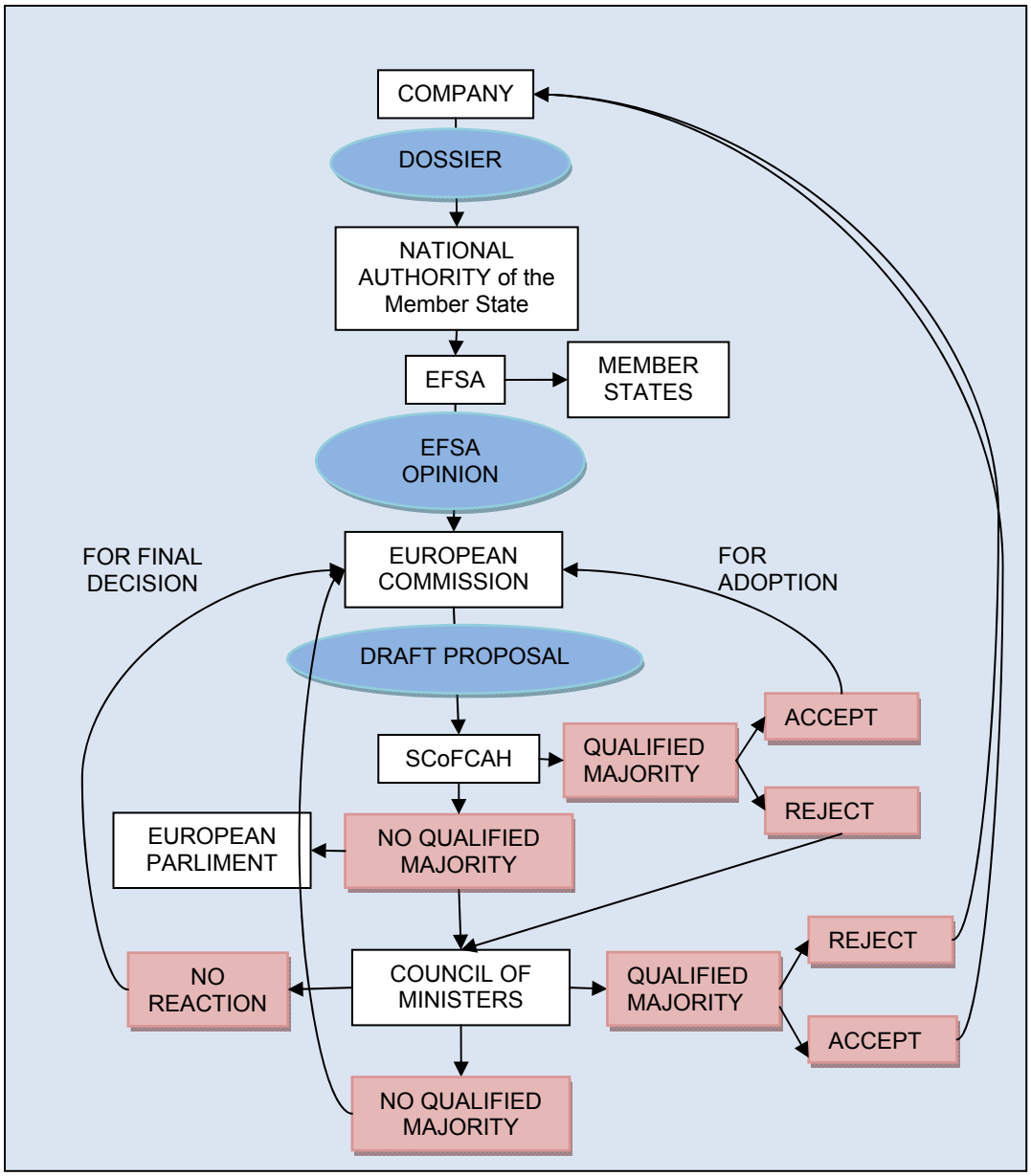

Figure 2.1 Schematic representation of the process of authorisation of GM food/feed following Regulation (EC) 1829/2003.

2.2.2 European Food Safety Authority

The European Food Safety Authority was established in 2002 under Regulation (EC) 178/2002 as the central authority of European Union (EU) risk assessment regarding food and feed safety (EC, 2002). The mission of EFSA is to provide independent scientific advice and clear communication on existing and emerging risks in food and feed, in close collaboration with national authorities and in open consultation with its stakeholders. More information is available at EFSA webpage: http://www.efsa.europa.eu.

EFSA's risk assessment work is performed by ten scientific Panels of independent experts. Panels are divided in different areas of the food and feed chain and supported by a Scientific Committee. The Panel on Genetically Modified Organisms (GMO Panel), supported by the GMO Unit, provides independent scientific advice on the safety of:

- Genetically modified organisms (GMOs) such as plants, animals and micro-organisms, on the basis of Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms
- Genetically modified food and feed, on the basis of Regulation (EC) No 1829/2003 on genetically modified food and feed.

The GMO Panel carries out risk assessments of these products in order to produce scientific opinions and advice for risk managers. The Panel carries out much of its work in the context of authorisation applications, both in writing opinions and in providing guidance documents that assist companies and other organisations in the preparation and presentation of applications. More information about the work of the GMO Panel is available in the EFSA webpage, through the link: http://www.efsa.europa.eu/en/panels/gmo.htm.

2.2.3 GMO legislation in other countries

GMO legislation in many countries is based on the same principles of assuring safety for human and animal health and for the environment. However, the practical approaches can vary from country to country. The GMO definition can be based on the technical process to obtain the organisms, like in the EU, Argentina, South Africa and Japan, or just on the final product, like in Canada (Lusser and Rodriguez-Cerezo, 2012). For instance, Canada did not create a new legal framework with the introduction of GMOs in market, but continued using its regulation on products with novel traits, including GM products. The focus is then clearly on the new traits possessed by the GM products and not on the technological process applied to obtain them.

In the US, three federal agencies have primary responsibility for the regulation of the products of agricultural modern biotechnology, depending on the intended use of the crop: The Food and Drug Administration (FDA), which has responsibility for the safety of food, feed and drugs, the US Department of Agriculture (USDA), which has responsibility for the safety of animal products and for the regulation of potential agricultural plant pests and noxious weeds, and the Environmental Protection Agency (EPA), which has responsibility for the correct use of

pesticides. Depending on its characteristics, a product may be subject to review by one or more of these agencies.

Since no specific laws were created in the US to regulate the products of biotechnology, these products are regulated under the same law (at least ten different laws), agency regulations and guidelines that cover products as food, animal feed, human and animal drugs and biologics, pesticides, plant pests and toxic substances (Pew, 2001).

2.2.4 The regulatory costs for breeding GM plants

The whole process of GMO authorisation is generally very demanding in terms of time to invest and costs, independently of the country. However, some legislation systems are more demanding than others. The reason can rely on the legislative system, on strong public opinion, on particular traditions, etc. For instance, the EU system requires labelling and traceability of GM products, unlike the US and Argentina. Also the EU requires a separate assessment for "stacked" GMOs, i.e. organisms that carry more than one gene (or trait) transferred from another organism. In the US "stacked" events are considered as authorised if the single events have been already approved.

Several studies have analysed the average time and costs related to the authorisation and marketing of GM plants. McElroy in 2003 estimated that the regulatory approval costs for each GM product in the US was $5-10 million in the 1990s and increased to $20-30 million after one decade. This increase is apparently due to more detailed requirements in the regulatory studies, especially for international approval (including approval in the EU) (McElroy, 2003). According to Redenbaugh and McHughen (2004), some agronomic seed companies budget $50 million for the full commercialisation of a new GM crop, in addition to the standard costs for developing and marketing a traditional variety.

Kalaitzandonakes et al (2007) produced a very detailed analysis of the compliance cost for the full regulatory approval of a GM maize with traits of insect resistance and herbicide tolerance. The authors estimate the cost range of all operations involved in the regulatory process of GM maize, including compliance costs related to specific requirements of some countries. For instance, the costs of getting approval for import to the EU were estimated between $230,000 and $405,000. According to this study, the total compliance costs for approval of GM maize vary between $7,060,000 and $15,440,000 for insect resistant maize and between $6,180,000 and $14,510,000 for herbicide resistant maize. According to Kalaitzandonakes et al., four cost

categories dominates in the total calculation of compliance cost for commercialisation of GM products and represent 60% of total costs. These categories are i) production of tissues, ii) compositional assessment, iii) protein production and characterisation and iv) molecular characterisation.

A recent study of the industrial organisation CropLife International (McDougall, 2011) reports a detailed evaluation of cost and time associated to the different phases of discovery, development and authorisation of a new GM crop. According to a survey of the six major biotech crop developers, time and cost of bringing a biotech crop to market has risen dramatically. The cost of discovery, development and authorisation of a new plant biotechnology trait introduced between 2008 and 2012 is estimated around $136 million, while the time from the initiation of a discovery project to commercial launch is 13.1 years on average for all relevant crops.

In general the order of magnitude for costs is always around tens of millions of US dollars, and the time calculated is never below two-three years. This analysis clearly indicates that releasing GM products into the market is not affordable for all kind of institutions. In particular, small and medium size enterprises and Universities cannot generally afford to invest such amounts of money, in addition to the uncertainty regarding the time of commercialisation. That would explain the current scenario of big enterprises commercialising GMOs worldwide (McElroy, 2003). Additionally, several authors agree that the high regulatory costs for GM crops and the strict requirements of the EU market for GM products, limit drastically the investments in developing GM fruits and vegetables (McElroy, 2003; Redenbaugh and McHughen, 2004). Big companies investing in agro-biotechnology are more oriented towards field crops, like GM soybean, corn and cotton, than fruits and vegetables.

2.3 Regulatory status of NPBTs

2.3.1 The new techniques working group (NTWG) in the EU

At the request of Competent Authorities (CAs) of EU Member States, the EC set up a specialised Working Group, NTWG, in December 2008 to evaluate a list of eight new techniques proposed by the CAs. Member States have each appointed scientific experts to participate in the work of the Group, which is assessing if the use of these new techniques should be considered to result into GMOs or GMMs (Genetically Modified Microorganisms) as defined under Directive 2001/18/EC

or Directive 90/219/EEC, respectively (EC, 2008). The following techniques were identified as the starting point for the consideration for the NTWG, by the Competent Authorities:

1. Oligonucleotide Directed Mutagenesis (ODM)
2. Zinc Finger Nuclease Techniques (ZFN) (comprising ZFN-1, ZFN-2 and ZFN-3 as defined in this study)
3. Cisgenesis (comprising Cisgenesis and Intragenesis)
4. RNA-dependent DNA methylation (RdDM)
5. Grafting (on GM rootstock)
6. Reverse Breeding
7. Agro-infiltration (comprising agro-infiltration *sensu stricto*, agro-infection and floral dip)
8. Synthetic Biology

While grafting, reverse breeding and agro-infiltration exclusively refer to plant breeding techniques, the other five techniques can be applied to both microorganisms and plants. The NTWG is evaluating them for both scopes.

This Thesis takes as starting point the list established by the NTWG, but Transcription Activator-Like Effector Nuclease (TALEN) techniques, Meganuclease (MGN) techniques and Early Flowering induction were added to the list, as we considered them relevant in modern plant breeding and since they also pose the question of whether to be in or out the scope of GMO legislation. On the other hand, agro-infiltration and synthetic biology are not considered in this study. Synthetic biology, as defined by the NTWG, is not considered as yet developed in plant breeding. Agro-infiltration is not considered as a new technique, since it was developed almost 30 years ago (Grimsley *et al.*, 1986). Additionally, literature and patent information reveals that the technique of agro-infiltration is mainly used for research purposes and in any case the agro-infiltrated plant is not usually further propagated in commercial plant varieties. Thus its legislative status does not seem to be relevant in commercial plant breeding. For these reasons, these two techniques were excluded from this study.

The EU NTWG has finalised its work. The resulting report has been sent to EU member states and is not yet made public. In parallel, EFSA was required to produce a scientific opinion on the safety assessment of each technique and already published the opinion on the safety assessment of plants developed through cisgenesis and intragenesis (EFSA, 2012). More details about EFSA opinion are provided in section 6.3.3.

2.3.2 Regulatory developments for NPBTs in other countries

EU Member States are not the only countries currently dealing with the legislative status of NPBTs. The JRC-IPTS organised a workshop in September 2011 to discuss the regulatory approaches for NPBTs in different countries (Lusser and Rodriguez-Cerezo, 2012). The workshop brought together experts from the following countries: EU, Argentina, Australia, Canada, Japan, US and South Africa.

The outcome of the workshop was that most countries are still in the process of evaluating the legislative status of NPBTs and did not reach final conclusions yet. Some countries just started to think about these techniques, since they did not receive yet any application for authorisation of products of NPBTs. The proceeding of the workshop (Lusser and Rodriguez-Cerezo, 2012) illustrate that some countries are foreseeing the exclusion of some specific techniques from the GMO legislation, like ZFN-1, negative segregants and cisgenesis (but only in the very strict sense of definition). However, they are not to be taken as final decisions.

Canada seems to have so far the clearest approach towards regulation of NPBTs. As for GMOs, Canada will continue using its legislation on plants with new traits, judging if the products of NPBTs possess new traits or not, compared with previously assessed plants, independently on the tools used to obtain them.

NPBTs seem to be still under evaluation by the US regulatory system. The USDA made publicly available its case-by-case evaluation of specific inquiries received about products of NPBTs: http://www.aphis.usda.gov/biotechnology/reg_loi.shtml. Table 2.1 schematically reports the responses of USDA about products of NPBTs, as ZFN 1 and MGN 2 (not considered as regulated), MGN 2 (to be evaluated on a case-by-case basis), Cisgenesis and Intragenesis (regulated only if *Agrobacterium* is employed in the plant transformation process). Finally, negative segregants, such as the products of RdDM and Early flowering techniques, are not considered as regulated articles if the absence of transgenes is carefully checked by means of molecular tools.

Table 2.1 Inquiries to USDA about the regulation of certain products of NPBTs

COMPANY	TECHNIQUE	PRODUCT	USDA response to inquiry
Dow Agrosciences	ZFN-1	Maize with reduce phytate content	Not regulated articles
Cellectis Plant Sciences	MGN 1 and 2	Plants with resistant to pathogens (MGN 1) and with herbicide tolerance (MGN 2)	MGN 1 - not regulated MGN 2 - case-by-case evaluation on specific inquiries
Wageningen University and Research Centre	Cisgenesis through *Agrobacterium*	Apples resistant to apple scab	Because of the use of *Agrobacterium* (a plant pest), these products are regulated by USDA
Mid-Florida Research and Education Center at the University of Florida	Intragenesis through biolistics	Red-berried seedless grapevine	Not regulated articles
University of Nebraska	RdDM	Sorghum bicolour with modified growth	Not regulated articles (only GE parent plants would need authorisation to be released)
North Carolina State University	Early flowering	Accelerated breeding of tobacco	Not regulated articles
Agricultural Research Service (ARS), Kearneysville	Early flowering	Accelerate breeding of plum	Not regulated articles

2.3.3 Detection of products of NPBTs

The EU GMO legislation requires that for each GMO submitted to approval a detection method is provided. Therefore, it is essential to verify if the products of NPBTs can be detected. For this purpose, a "New Techniques Task Force" (NTTF) was established in the IHCP (Institute for Health and Consumer Protection) of the JRC to analyse the detection options for the products of the new techniques established by the NTWG (Lusser *et al.*, 2011).

According to their analysis, only for the techniques of ZFN-3, cisgenesis and intragenesis, it is possible to unambiguously detect the presence of the inserted gene, provided that previous information about the DNA sequence introduced and the neighbouring sequences is available. The detection would be performed through DNA-based methods, as usually for transgenic plants.

For products of targeted mutagenesis, the created mutation could be detected, with previous knowledge of which mutation to look for. However, there would be no way to differentiate if the mutation was created by one of the NPBTs, by classical mutagenesis or if it is a spontaneous mutation.

Regarding RdDM, there are methods to detect changes in the methylation pattern, which could establish if a plant was epigenetically modified. Again, previous knowledge about the target

sequence should be available to direct the detection method. And also in this case, there would be no possibility of differentiating between the modification obtained by RdDM or by environmental effects.

Finally, the products of reverse breeding and early flowering, and the fruits of the non GM scions grafted onto GM rootstock, could not be detected, since the genome of the final products do not carry any mutations or foreign sequence.

Chapter 3. Intellectual Property in plant breeding

3.1 Introduction

According to the World Intellectual Property Organization (WIPO), intellectual property (IP) refers to creations of the mind, including inventions, literary and artistic works, and symbols, names, images, and designs used in commerce. IP is divided into two categories: industrial property, which includes inventions (patents), trademarks, industrial designs and geographic indications of source; and Copyright, which includes literary and artistic works.

IP system is theoretically a mean to encourage creative intellectual endeavour in the public interest. In particular, the patent system is supposed to encourage industry and innovation by rewarding inventions and protecting investment in product development (Dunwell, 2005). These are the reasons for which different types of IP systems are in force in several countries. Indeed, some scholars state that IP rights have a positive effect on cumulative innovation. However, on the other side, other scientists believe that the IP system prevents competition and therefore hinders further innovation in all fields (Murray and Stern, 2007).

IP in the field of plant breeding is partly related to the industrial property but has generally a *sui generis* character, due to the long and multifaceted history of plant breeding for mankind and also due to an emotional component associated to agriculture (Rodríguez-Cerezo, 2012). Two main forms of IP are used in plant breeding: plant variety rights and patents (Dunwell, 2005; Dodds *et al.*, 2007). Plant variety rights are established by the UPOV (UPOV, *Union Internationale pour la Protection des Obtentions Végétales*) convention (UPOV, 1991), as illustrated in next sections, and are applicable to basically all new varieties of plants. The level of protection conferred by plant variety rights varies depending on the specific national legislation based on UPOV that is applied in each country. Patent system in plant breeding was introduced mainly by the use of biotechnological tools, especially in the 1970s-1980s.

This chapter is meant to provide an overview of the historical background of intellectual property in plants, by describing how the different IP systems for plants have developed in parallel with the progresses in agriculture. Both the mechanisms behind plant variety rights and patents are illustrated, in order to give a clear overview of the differences between the two systems and also the differences of their application in different countries, in particular the EU and the US.

3.2 Historical background of IP in plant breeding

3.2.1 From plant domestication to IP in plant breeding

The modification of plant characteristics for human needs began in the Neolithic Age, with the change of human population from nomadism to sedentary lifestyle. Humans started selecting seeds from plants with favourable characteristics for agriculture and continued for hundreds of cycles of plantation and harvesting. In this way, through several plant generations, the plants selected by humans became more and more genetically distant from their wild counterparts, from which the selection started. This process of accentuating traits that benefits humans is called plant domestication. The genetic differences between domesticated plants and wild counterpart are due to the preference of the human population towards "non-natural" characteristics (e.g. like the adhesion of seeds to the plant) and the parallel fixing of new, favourable mutations. A consequence of this process is that domesticated plants would not survive in a natural environment, since they lost their main wild characteristics (Cubero, 2003).

Plant domestication evolved during centuries, applied in particular to a limited number of plants that constitute today our agricultural heritage. Some historical facts accelerated the progress in plant breeding, until the more sophisticated technical developments of the last decades. In particular, agriculture was developing during the Seventeenth and Eighteenth centuries due to the scientific progress driven by the Age of Enlightenment, to the increase of the population in Europe, to the import of new plant varieties, especially from America, and to the activity of seeds commercialisation (Rodríguez-Cerezo, 2012).

The progress in the knowledge of the genetic basis of heredity in plants gave a further boost to the practice of plant breeding. In the second half of nineteenth century, Gregor Mendel deciphered the mechanisms of genetic heredity and in 1865 published the paper "Experiments on Plant Hybridization" (Mendel, 1865). The improved knowledge about the genetics of plants and plants' heredity established the scientific basis of the plant breeding process, thus plant breeding became systematic.

A parallel phenomenon to the progress of plant breeding was the growing importance of the private sector in agriculture, due primarily to the growing role of seed industry. This tendency increased at the beginning of the twentieth century. In the 1920s the commercial seed market

grew dramatically with the development of the first hybrid maize seed (Duvick, 2001). This is considered as a crucial event both for agriculture and for IP.

Some plants, like wheat, are able to self-pollinate. Therefore, the breeder can obtain pure wheat lines (with all traits in homozygosis) just by self-crossing the plants for a few generations. This implies that these lines can be easily reproduced by farmers or breeders simply by self-crossing them again. On the other side, some plants, like maize, are not cross-compatible thus do not naturally allow the production of homozygous lines.

The technical progress in plant breeding has resulted in the obtainment of very productive and homogeneous lines also from self-incompatible species. Inbred maize lines are created by artificially induced auto-fertilisation. The two inbred lines constitute the parental lines, which are usually weak and not very productive. The parental lines are crossed to obtain a very homogeneous and productive hybrid seed (considered as first generation or F1) (Cubero, 2003). The hybrid seed is usually very vigorous and productive, thanks to the phenomenon of "heterosis", which refers to the increase in size or rate of growth of offspring over parents.

An additional aspect of the hybrids is that the offspring (F2) of the hybrid F1 seeds is a very heterogeneous population, because of the high level of heterozygosis of hybrid seeds. Characteristics of the parental inbred lines appear again, thus the productivity of this next generation is not comparable to the hybrids. Consequently, farmers would have little interest in growing hybrid seeds' offspring and would also not be able to reconstitute the parental inbred lines. Therefore, in addition to the progress in the productivity of maize, hybrids were offering a natural protection against the appropriation or copying of seed varieties. It can be considered as a natural form of IP protection (Rodríguez-Cerezo, 2012).

Because of their characteristics of defence against copy, hybrid seeds attracted in particular the private sector in a time of absence of legal instruments for intellectual protection of crop varieties. The success of hybrid maize seeds encouraged the application of the technique for the obtainment of other hybrid crops, which was not always technically feasible. On the other side, self-pollinating crops were not that attractive for the industry given the ease with which its seeds could be reused by farmers and thus they remained primarily in public research.

Despite the advantages of hybrid technology, the emerging seed industry was still demanding for legal instruments that would meet their needs of protection of their products against competitors. The natural protection conferred by hybrid technology is not absolute and additionally seed industry was also interested in exploiting self-pollinating seeds and crops that propagate vegetatively (through shoots or roots). The development of IP system in plant breeding followed a different route compared to production of other industrial commodities,

resulting in a *sui generis* system. The next paragraphs summarises the development of instruments for the protection of agricultural crop varieties.

3.2.2 Townsend-Purnell Plant Patent Act (PPA)

The introduction of patent protection in agriculture is generally associated to the introduction of biotechnology tools in the 1980s. However, the first instrument of IP in plant breeding was a plant patent law designed in the US in 1930 according to the needs of breeders, which were asking for a way to secure control on their products since the end of the nineteenth century. This law introduced the concept of "plant patent" to be distinguished from utility patents, which already existed for industrial activity (Dodds *et al.*, 2007).

The Townsend-Purnell Act of 1930, still in force, allows the patenting of plant varieties of asexual reproduction (vegetative), but excluded the protection of crop varieties of sexual reproduction (seeds). This patent system is currently available in the US and covers many varieties of ornamental crops, of strawberries, fruit trees (especially citrus) and ornamental trees.

3.2.3 UPOV convention

To provide an international response to the urgent need for IP protection of plant varieties, the UPOV convention was adopted in Paris in 1961 (UPOV, 1961). The convention establishes the minimum standards of a *sui generis* IP system adapted to the characteristics of plant breeding, with the aim of encouraging breeders to develop new crop varieties. It also describes the general parameters to develop national systems (rights, exemptions, requirements, duration). The rights of plant breeders take different names according to the laws of each country: Plant Variety Protection Certificate, Título de Derecho del Obtentor, Certifique d'Obtention Vegetal, Privativa per Varietà Vegetale, etc.

The convention of 1961 was revised in 1972, 1978 and 1991 (UPOV, 1991). The UPOV Convention of 1978 provided for the first time the protection of plant varieties as a special form of industrial or intellectual property. The breeders can grant a right on a crop variety that meets the four criteria of being new, distinct, uniform and stable. The UPOV reviews have led to a gradual strengthening of the position of breeders and a limitation of the exemptions in favour of the farmer. Additionally, the number of agricultural species eligible for protection was extended in the different versions until covering all crops in the act of 1991 (Dodds *et al.*, 2007). UPOV

1991 act has been adopted by most industrialized countries including the US and the EU. However, UPOV 1978 version is still in force in some other countries.

In general, the UPOV convention provides two exemptions: i) a breeder exemption or privilege (also called research exemption) and ii) a farmer exemption or privilege (Dodds *et al.*, 2007). These exemptions differentiate plant variety rights from utility patents. Farmer's exemptions refer to the possibility for farmers of saving seeds for the sole use of replanting their land, but not for selling or trading them. This exemption is not considered obligatory in the UPOV 1991 act but each member state of UPOV can decide whether and how to maintain it in its legislation.

Breeder's exemption allows breeders to use the protected variety in their breeding process to develop a new plant variety. UPOV 1991 version also reduces the breeder exemption. The rights conferred by the plant variety protection are extended also to the so called "essentially derived varieties", meaning those varieties that present only relatively minor changes compared to the original variety (Dodds *et al.*, 2007). This means that a protected plant variety which is transformed for the insertion of a new gene can be considered as essentially derived, unless the gene provokes big changes in the phenotype. Therefore, the institution that produces the transformed variety would need to license the protected plant variety before to commercialise the resulting product. The use of protected varieties for basic research with no commercial purposes is unconditionally allowed.

The definition of essentially derived varieties and the consequent legislative measures implied have often been subject of juridical disputes between breeders. However, it is highlighted here that the legal gap caused by that definition is not the subject of this study and therefore, it will not be detailed further. Another legal gap in the field of plant breeding protection is determined by patents filed in the EU for the protection of gene sequences of interest for plant transformation. As explained in next section, plant varieties are not patentable subjects in the EU. However, by patenting potential transgenes of interest, the protection could consequently be claimed for transformed plants carrying those genes. This case was also cause of contentions and will not be detailed in the scope of this study.

The US adopted the UPOV convention with the PVPA (Plant Variety Protection Act) (USDA, 2006). The first version was issued in 1970 in correspondence to UPOV 1961, while UPOV 1991 was adopted in PVPA 1994. As said before, this last version limited farmers and breeders privilege, which were previously very broad. Because of so broad exemptions in the previous versions, traditional plant breeder's certificates based on the UPOV Convention were considered as a weak form of protection in the US. This could be one of the reasons for the interest that US seed companies always had in protecting their plants through patents according to the PPA (section 3.2.2) when possible.

EU member states adopted UPOV agreements between 1960 and 1970 and introduced the corresponding regulations in their national legislation. The EU integrated the adoption of the principles of the UPOV Convention in 1991 by the Council Regulation (EC) 2100/94 on Community plant variety rights (EC, 1994). The regulation creates a form of European protection that coexists with national protection systems. However, the two systems are not compatible: any variety which is subject matter of a Community plant variety right shall not be the subject of a national plant variety right, thus the breeder must choose which title to claim.

The European Community Plant Variety Office (CPVO) was founded for this purpose in Angers (France) (http://www.cpvo.europa.eu/main/). National offices (like the Plant Variety Rights Office in the UK or the Spanish "Oficina Española de Variedades Vegetales") can also process a Community application that can be submitted in one of the eleven official EU languages.

According to this Directive, breeder's exemption is guaranteed, with the limitation described previously of essentially derived varieties. Farmer's exemption is allowed, but limited to certain species and only if used freely by small farmers (defined in the Regulation), while the rest of farmers must provide financial compensation to the breeder if they want to benefit from the exemption. In the US the farmer's exemption remains theoretically greater, and this makes plant breeding rights stronger in the EU than in the US. This can constitute another reason to explain why US is more oriented in the patenting of plants, compared to the EU.

3.2.4 The advent of biotechnology in plant breeding

From 1980s on, biotechnology tools were incorporated in plant breeding, in particular thanks to the progress of plant transformation techniques and genome sequencing, which increased the use of molecular markers for plant selection. These technical developments launched the introduction of the patent system in plant breeding, also due to the pressure of the industry for protecting their biotechnological applications in plants. Therefore, in the 1980s and 1990s the patentability of plants is incorporated into the legislation of most developed countries (Fleck and Baldock, 2003).

The protection of plant varieties was raised in the TRIPS agreement (Agreement on Trade-Related Aspects of Intellectual Property Rights) of 1994, signed by members of the World Trade Organization (WTO). The agreement states that all signatories are obliged to provide protection to plant varieties "either by patents or by an effective *sui generis* system or by any combination thereof" (WTO, 1994). The patentability of living organisms was excluded in most countries. In the EU, plant varieties are explicitly excluded as an object of patents. By contrast, in the US the

concept of patents on plant varieties already exists since the PPA of 1930. This represents an additional reason for the general preference of the patent system by US breeders.

In 1998 the EU Parliament published the Council Directive 98/44/EC on the legal protection of biotechnological inventions (EC, 1998), with the objective of clarify what is patentable and what must be excluded from patentability. According to the Directive, inventions are patentable if they are new, involve an inventive step and are susceptible of industrial application, even if they concern a product consisting of or containing biological material. Directive 98/44/EC excludes from patentability i) the human body or one of its elements, including human DNA sequences, ii) plants and animals varieties, as described before, and iii) essentially biological processes for the production of plants or animals, such as crossing and selection.

The Directive also establishes the concept of compulsory cross-licensing. This means that if a breeder needs a patented invention to acquire or exploit a plant variety, he may apply for a compulsory licensing for non-exclusive use of that invention, by paying an appropriate royalty. Conversely, if the biotech patent holder cannot exploit its invention without infringing plant variety rights, he may request a compulsory license to exploit the variety.

3.3 Plant variety registration

3.3.1 Plant Variety definition

A plant variety is defined by the UPOV convention (Article 1(vi)) as follows:

"a plant grouping within a single botanical taxon of the lowest known rank, which grouping, irrespective of whether the conditions for the grant of a breeder's right are fully met, can be

- defined by the expression of the characteristics resulting from a given genotype or combination of genotypes,
- distinguished from any other plant grouping by the expression of at least one of the said characteristics and
- considered as a unit with regard to its suitability for being propagated unchanged;"

According to Reg. 2100/94, Community plant variety rights shall be granted for varieties that are distinct, uniform, stable and new (EC, 1994). Therefore, the applicant for registering a new variety must demonstrate that the variety fulfils these requirements.

Distinctness means that the characteristics expressed by the variety, which results from a particular genotype or combination of genotypes, are clearly distinguishable from any other varieties that are in the common knowledge. The characteristics for which this criterion is applicable can be morphological, agronomic, cytological, chemical, etc.

Uniformity means that all the individuals of the variety express the characteristics of distinctness in the same way. A certain level of variability is admitted, since the perfect uniformity is very complicated to reach, especially for plants that are not self compatible.

Stability refers to the stability of the characteristics with the time and after propagation of the plants.

Finally, novelty means that the variety is not already commercially available at the date of the application for the plant variety rights.

Moreover, the variety must be designated by a denomination in accordance with the provisions of Article 63.

3.3.2 Application for Plant Variety Rights

The application for registering a new plant variety must contain complete legal information about the applicant and the variety. The application also includes a thorough description of the variety demonstrating that it meets the requirements of novelty, distinctness, uniformity and stability. Additionally the applicant has to provide a certain amount of plant material of the plant variety to be registered, to allow the examiners to confirm the established criteria of novelty, distinctness, uniformity and stability. All required trials of technical examinations are entrusted to competent bodies. The trials on average are conducted over a two year period in accordance with protocols established by the CPVO and monitored by its technical experts. Accordingly, varieties submitted are compared with existing varieties of the same species.

According to Regulation 2100/94, Community plant variety rights are in force for 25 years or, in the case of varieties of vine and tree species, for 30 years, after the year of grant. The Council, acting by qualified majority on proposal from the Commission, may, in respect of specific genera or species, provide for an extension of these terms up to a further five years.

The CPVO keeps a register of the applications for Community Plant Variety Rights in the Official Gazette, which contains all the applications together with statements of the taxon and the

provisional designation of the varieties, the date of application and the name and address of the applicant, of the breeder and of any procedural representative concerned, proposals for variety denominations, among other information.

In accordance to Regulation 2100/94, the CPVO charges fees for its official acts as well as for each year of the duration of a Community plant variety right. The office provides detailed information about the fee required for each plant species in its website: http://www.cpvo.europa.eu/main/en/home/methods-of-payment/applicable-fees/list-of-species-and-their-fee-group. The average time associated to the registration of a new plant variety is around two-three years and the average cost is of 10.000€.

3.4 Patents in plant breeding

3.4.1 Patents in general

A patent is an exclusive monopoly right granted to an invention for a limited amount of time. The grant prevents others to making, using, selling, or distributing the invention without permission of the patent owner. The procedure for granting patents, the specific requirements, and the extent of the exclusive rights vary between countries according to national laws and international agreements. There are however common patentability criteria required to grant patent protection to an invention. According to the European patent law (EC, 1998), the invention must be new (which corresponds to the criteria of novelty in the US), susceptible to industrial application (concept of utility in the US) and must involve an inventive step (concept of non-obviousness in the US). Typically, the information disclosed in a patent must be sufficient to enable a person skilled in the field to reproduce the invention (EC, 1998).

A patent application can be filed in one or more national patent offices (like e.g. the Japanese Patent Office – JPO - or the UK Intellectual Property Office – IPO), can be filed at regional level (e.g. at the European Patent Office (EPO)) or can be filed through the PCT (Patent Cooperation Treaty, administered by WIPO) route as an international patent application, which is intended to ultimately result in one or more granted national or regional patents. These options can be applied simultaneously or sequentially to the same invention. The very first filed application, whether filed as a national, regional or PCT application, is known as the priority application and is given a "priority date". Subsequent applications filed typically to expand the geographical scope of protection have a common priority date, and are members of the same patent family (Martinez, 2010).

After filing of the patent application, the patent office examines the criteria of patentability and decides whether to grant the patent or not. Patent monopoly according to the EU legislation starts form the time of patent filing and lasts 20 years. The US has now adopted the same criteria, but before 1995 it counted 17 years from the date of patent grants. The time between filing and granting can range from two-three years to more than 20 years (Dunwell, 2005). According to a recent OECD report (Agrawala *et al.*, 2012), the cost of filing a European patent was estimated at 30,530€ in 2004 and the average time before granting was 40.6 months in 2005. If a patent application is filed at additional patent offices, the costs raises proportionally with the geographic coverage.

A published patent application usually shows a section of bibliographic information including the title of the invention, the name of the inventors and of the assignees (normally the institutions to which the inventors belong), the list of members of the same patent family and the abstract of the invention. Different dates are associated to a patent application and appear in the bibliographic information: usually the priority date (date of first filing), the publication date, and the dates of filing of other family members. Additionally there is a section dedicated to the patent description, which is a very detailed text containing all possible information that allows a skilled person in the field to reproduce the invention. The description is usually written in a complex language typically drafted by trained legal experts. The description includes background information, technical details of the invention and references to the scientific literature and to previous patents.

An invention described in a patent application might include steps that belong to pre-existing granted patents or patent applications. This means that the realisation of the described invention for commercial scopes would need the licensing of those patents, which must therefore be cited in the description. Some patents might be cited just as additional information in the background information provided in the patent application. Patent citation analysis is a useful method to define the transfer of knowledge among patents and to identify key patents on a specific subject: the ones that are cited the most, probably due to a broader content (Johnson and Lybecker, 2012).

A very crucial section of a patent application is the section of claims. Patent claims are part of the patent application in which the applicant specifies exactly the scope of protection of the invention, supported by detailed disclosure of the invention. The number of claims vary in all patents and usually follow a hierarchical structure in which some claims (like the number 1) are independent and dominant, while the others are linked to the dominant claims (and are therefore dependent). During patent examination, the examiners determine if all claims are valid

or if some claims are not accepted and have to be eliminated, together with all dependent claims below.

In the field of plant breeding, patent claims for an invention can cover: new varieties (only in the US), transgenic plants and descendants, groupings, particular traits, parts, components (e.g. specific genes), products (e.g. fruit, oils, pharmaceuticals), material used in industrial processes (e.g. cell lines used in cultivation methods), reproductive material (e.g. seeds or cuttings), culture cells, breeding methodologies, vectors and processes involved in the production of transgenic plants, etc.

Beyond their utility as tool to guarantee commercialisation, patents can be used by scientists as useful source of information, which is not necessary disclosed in scientific literature, also considered the more limited amount of text in papers compared to patents (Thangaraj *et al.*, 2009). The information disclosed in patents is up-to-date and oriented to industrial application, but the limited use of patent literature by the average scientist could be due to the complexity of patents in terms of legal language and. Compared to scientific literature, patents might provide more information about the developers, possible applications of a technique and fundamental technical details, since the methodological description in the text of the patents has to be as detailed as possible. Dunwell (2005) estimates that 30-40% of all DNA sequences are only available in patent databases and states that this information is often ignored by academic scientists. DNA sequences are fundamental information for the realisation of most methods in plant biotechnology.

3.4.2 Patents in plant breeding

As stated in Directive 98/44/EC, "natural processes" like crossing and selection are not considered as patentable subjects. Therefore, in plant breeding, the patentability is particularly relevant in those methods that make use of biotechnological tools, like in vitro fertilisation, marker assisted selection or plant transformation. Plant transformation requires a very complex protocol, in which several steps can be patent subjects, including materials used, microorganisms involved and DNA sequences of genes, regulatory sequences, vectors and so on. Table 1 shows the number of patents related to genetic transformation in the US between 1979 and 2000 (Dunwell, 2005).

Table 3.1 Summary of granted US utility patents (1979–2000) in the category 'genetic transformation'. Some patents are included in more than one subcategory (Dunwell, 2005)

Subcategory	Patents (no.)
Transformation platforms	928
Mutagenesis	153
Genetic markers	624
Selectable marker techniques	486
Culture growth, cell differentiation, etc.	1632
Transformation stability/heritability	33
Diagnostic techniques	1399
Total	4129

Patents in plant breeding might claim a process, like e.g. a method to obtain plant transformation, a method to select transformed plants or a method to prepare the transformation vector. The patent subject can also be a product, like the transformed plant (or plant variety in the US), but also a specific vector, a transformed strain of *Agrobacterium* or a new machine for biolistic transformation. Often patent claims include a mixture between processes and products (e.g. the transformation method and the transformed plants).

The high number of patented steps in the protocols of plant transformation reduces considerably the possibility of obtaining a transgenic plant without infringing any previous patent. The issue of freedom to operate will be analysed in more details in the section 3.4.3.

As described in the previous sections, different forms of protection can be applied to crop plants depending on the specific case and on the national requirements. Sometimes different forms can be applied simultaneously. These rights include in particular plant variety protection (according to the UPOV convention), plant patents and utility patents. Other rights can be also included in plant breeding, like trademarks, trade secrets and genebank management (Dodds *et al.*, 2007), but are not within the scope of this study.

Regarding national requirements, as already mentioned, the main difference between the US and the EU is that EU patent legislation forbids claiming specific plant varieties in patents, while in the US this is allowed. This means that in the EU a plant transformation method can be patented only if it is shown to work in a taxon above the "plant variety" taxon.

The granting of a patent does not automatically means that the patent subject can be placed into the market. Beyond patent rights other legal requirements that are independent from the patenting process need often to be covered (Fleck and Baldock, 2003). In the case of patents for plant transformation, for instance, the transgenic plants obtained cannot be commercialised until all legal requirements about GMOs are covered. In the EU this would mean until the EC gives the authorisation for the commercialisation of those GM plants. If the patent refers to a plant breeding tool that is outside the scope of GMO Directive (e.g. mutagenesis through chemicals), then the new pant varieties obtained should be registered before to be put into the market.

3.4.3 Freedom to operate (FTO)

Freedom to operate refers to the ability to exploit a technical process or product (for research or commerce) without infringing any intellectual property rights. The analysis of the level of freedom to operate associated to a specific protocol, e.g. of plant transformation, requires two steps: product deconstruction and product clearance (Kowalski *et al.*, 2002). Product deconstruction refers to the definition of the technical content of the product and its deconstruction in all the ingredients, processes and combinations thereof used to obtain the final product. In the case of plant transformation, this would also include DNA sequences (like genes, promoters and markers), methods of transformation and selection, vectors employed etc. Product clearance refers to assemble and document all information on intellectual property associated to the steps identified: patents, licenses, material transfer agreements etc (Kowalski *et al.*, 2002). This allows identifying IP infringements that the product developer must remedy to place the product on the market.

"Experimental use exemption" refers to the possibility of using patented inventions in basic research without licensing them, under the condition that the inventions are not put to routine use and that the final objective is not the commercialisation of the research results (Dunwell, 2005). This exemption is not always completely clear but generally allows researchers to carry out their projects without the need to license every tool they employ. On the other hand, in case they decide to place into the market what they obtain in the experiments, they have to consider their freedom to operate and perform the analysis as previously described in order to acquire all needed licenses or agreements.

The case of Golden Rice (Ye *et al.*, 2000) provides an example of a highly fragmented protocol, in which patented steps are owned by around 40 organisations. Golden Rice refers to GM rice

transformed with two beta-carotene biosynthesis genes in order to express high level of beta-carotene (pro-vitamin A) in the edible part of the plant. The scope of the production of Golden Rice is to feed population of developing countries in which rice is a staple food and there are not available sources of vitamin A. These populations suffer of severe vitamin A deficiency that causes blindness (Stein *et al.*, 2006). According to Kowalski (2002), more than 70 patents must be licensed in order to enable the commercialisation of Golden Rice. Due to the humanitarian purpose of the cultivation of Golden Rice, patent owners involved in the process are being requested to freely license their inventions (more information is available in the dedicated Web page: http://www.goldenrice.org/Content2-How/how9_IP.html).

Although much of the early development of plant breeding techniques was performed in universities, most of the patents are consolidated in the hands of a few private companies (Dunwell, 2005). According to Graff (2003), 24% of US granted patents in agricultural biotechnology belong to public institutions, a high percentage compared to any other IP sector. However, the public IP ownership is highly fragmented across several institutions and obstacles freedom to operate for the development of transgenic crop. On the other hand, the top 5 private corporations own 41% of US patents in agricultural biotechnology. This clear dominance of the private sector in IP ownership for the development of transgenic crop is another cause of the increasing improvement and cultivation of large market crops, such as soybean and maize, while work on crops of less commercial interest is progressing slowly, in the hands of public sector (Atkinson *et al.*, 2003). Together with strict regulatory requirements and low public acceptance, IP constraints are considered as another important factor limiting the development of new transgenic crop varieties (Atkinson *et al.*, 2003).

Certain initiatives are meant to improve the freedom to operate of the public sector in the field of agricultural biotechnology. The rule of compulsory licensing was established already in the Paris Convention of 1883 to prevent the abuses which might result from the exercise of the exclusive rights conferred by the patent (Paris Convention, 1883). However, licensing fees can be often a limiting factor for the budget of public institutions. Non-profit organisations have been founded in order to strengthen the IP power of public sector in plant biotechnology and to help developing countries to access patented technology. An example of those organisation is PIPRA (Public Sector Intellectual Property Resource for Agriculture), which collects more than 50 members institutions from more than 15 countries around the world. More information is available here: http://www.pipra.org/.

Nevertheless, it must be considered that patent protection generally lasts for 20 years. Therefore, the hegemony of private sector in plant biotechnology and seed industry will be

challenged when the main patents start to expire. The earliest patents on GM crops will expire in 2014 (Gruskin, 2012), in particular the patent of Monsanto for Roundup Ready herbicide tolerant soybean. We can thus expect the development of "generic" GM plants by other companies or by the public sector. However, the development of generic versions of GM plants is not straightforward since it will require re-submission or legal access to original safety testing data, along with any new information on safety that updated legislation may require. It is far from clear how seed breeders intending to develop generics and biotech companies will solve these issues.

3.4.4 Patent licensing

Licensing means that a patent owner (the licensor) grants exploitation rights over his patent to a licensee. Different types of agreements can be made between licensor and licensee about the type of rights conferred by the license and the type and the amount of payment requested. Generally the underlying terms are based on market considerations. Normally a cost-benefit analysis is performed by both parties for the negotiation of the license. If a license is required for the patent related to a product with promising commercial expectations, the licensor can negotiate for a high payment.

Patents can be licensed on an exclusively or non-exclusively basis. Exclusive license means that the licensor agrees not to grant other licenses with the same scope, field and geographical coverage. In plant breeding, this approach is typical for traits technologies (like gene sequences conferring new functionalities to plants), while enabling technologies (like plant transformation) are typically licensed non-exclusively.

In patent licensing, different types of payments are possible and can be combined in different ways, depending on the agreement conditions between licensor and licensee (OECD, 2006). Generally the following types of payment are considered:

- Up-front fee: is the payment made when the license agreement is signed, and in some cases is the only amount paid. For enabling technologies in plant breeding could be between $10.000 and $100.000 and for trait technologies between $30.000 and $100.000 (Lars von Borcke, personal communication). In some cases, the up-front license fee is already included in the cost for sale of specific equipments or reagents, like it could be the case of Taq poymerase and thermocyclers for PCR.

- Milestone fee: is the payment that the licensee must make if certain events (i.e. milestones) occur, e.g. after proof of concept, on the first field trial or commercialisation. For enabling technologies is estimated between $20.000 and $200.000 and for trait technologies between $20.000 and $500.000 (Lars von Borcke, personal communication).

- Annual fee: For enabling technologies is estimated between $5.000 and $30.000 and for trait technologies between $15.000 and $50.000 (Lars von Borcke, personal communication).

- Annual royalty: is usually calculated as a percentage of gross or net sales and it is paid weekly, monthly or quarterly, depending on the specific agreement. Usually it is not commanded for enabling technologies while for trait technologies can be calculated as the 1 to 3% on net sales or on trait fee (Lars von Borcke, personal communication).

The approaches licensors take on licensing agreements has a big impact on future research and development, in particular when involving fundamental or new technologies. Therefore, it is of primary importance that licensors follow best practices in licensing (OECD, 2006) not to propose prohibitive agreements to licensees that would hinder the technological progress in a specific field.

Chapter 4. Scope and objectives of the thesis

4.1 Scope

A number of NPBTs have been developed in the last decade (following advances in molecular biology) with the potential to become alternatives to conventional breeding and standard transformation methods resulting in transgenic plants. These NPBTs techniques include targeted mutagenesis, plant transformation with DNA sequences from cross-compatible species, grafting techniques in which the upper plant part does not carry any new DNA sequence and techniques in which transgenesis is employed only as intermediate breeding step and the final products are free of foreign genes.

These NPBTs are challenging the legislation on plant biotechnology in the EU and other countries. Due to the characteristics of NBPTs and the nature of the final products, it is unclear if they fit into the "legal" definition of GM technologies or GM plants. The uncertainty about the regulatory status of these new techniques creates a state of expectation among many developers of these techniques, especially small and medium size enterprises or academic institutions. Their decision about the investment for the commercialisation of the new plant products will be affected by the final outcome on the regulatory decision. In case those products are classified as falling within the scope of the GMO legislation, their developers would have to follow all the process for GMO authorisation, which implies a high investment in money and a high uncertainty about the time needed.

Currently, very little was known about the actual state of development of NPBTs, about the potential to become adopted by the breeding industry, about the regulatory questions they pose worldwide and about the distribution of research and commercial activities on these new techniques.

The general objective of this study is to analyse whether NPBTs have a potential in plant breeding. All activities worldwide related to research or commercialisation of new plant breeding techniques are mapped and their potential is analysed in terms of possible applications in plant breeding, drivers and constraints for their deployment and value as alternatives to pre-existing plant breeding methods. Overall it will be analysed how much their commercialisation is affected by regulatory and intellectual property issues. The scope of this study is further detailed in the following paragraphs, divided into three main objectives.

4.2 Objectives

4.2.1 State-of the-art of NPBTs

The first objective of this study is to define the state-of-the-art of the research in NPBTs in the EU and other countries, to determine their level of adoption by academy and industry and to identify main drivers and constraints for adoption of NPBTs from the points of view of technical, economic and regulatory issues.

4.2.2 Patent landscape analysis

The second objective of this study is to describe and examine the patent landscape on NPBTs that determines the commercial interest in this area. Through the patent landscape analysis, key industry players are identified in NPBTs and are described in terms of patent portfolio and ownership of key patents. For each NPBT the level of patent fragmentation is defined.

4.2.3 Comparative analysis

The third objective of this study is to perform a comparative analysis of costs, intellectual property barriers, time and quality between new and conventional plant breeding techniques. The comparative analysis is performed through specific case-studies in which the same breeding objective is achieved through different technical alternatives.

Chapter 5. Methodological approach

5.1 Literature search on NPBTs

5.1.1 Literature database and search method

The bibliographic database ISI Web of science was employed for the literature search on NPBTs since it is considered as one of the most comprehensive literature databases. The aim of the search was to build a list of all research papers or review papers (including commentaries, opinions and letters) about NPBTs published until the end of the year 2011. The techniques for which the literature search was carried out are the ones defined in section 1.4.

The literature search was performed through search keywords, specifically chosen for each technique. Boolean operators (AND, OR, NOT) and Truncation wildcards, like the asterisk * for the search of words of different length, were employed in order to refine the search. Quotation marks were used to find words that must appear adjacent to each other (i.e. "zinc finger nuclease"). For many techniques, keywords were used in combination with the word "plant" connected through the Boolean operator AND. Searches on individual plant name(s) were also carried out. However, in most cases, they did not provide additional results. In some cases, a search for authors' names was also performed with the aim of double checking the obtained results.

The list of search keywords employed in the literature search for NPBTs is presented in Table 5.1. Keywords that were discarded because of a lack of results are not presented. For example, ODM is also known under many other names, so different combinations of words were tested, but only some of them resulted in findings in the field of plant breeding.

The list of publications obtained for each technique was manually screened in order to select review papers or research papers describing the use of the technique for plant breeding. Non-relevant publications were eliminated. Review papers, including commentaries, opinions and letters, were kept in order not to loose information, since the general number of publications about NPBTs is quite low (23 on average per technique).

Table 5.1 Keywords employed in the literature database ISI Web of knowledge for the search of publications related to NPBTs.

Technique	Keywords
TARGETED MUTAGENESIS	
ZFN	"zinc finger nuclease" and plant* "zinc finger nuclease" and crop* ZFN and plant* ZFN and crop*
ODM	oligonucleotide* and mutation* and plant* oligonucleotide* and mutagenesis and plant* "oligonucleotide directed mutagenesis" "chimeric rna/dna oligonucleotide*" "chimeric oligonucleotide*" and plant* chimeraplasty and plant* "site-directed mutagenesis" and oligonucleotide* and plant* "gene targeting" and oligonucleotide* and plant* "rna/dna oligonucleotide*" and plant* GRON "gene surgery" "chimeric oligonucleotide-dependent mismatch repairs" OR cdMMR
MGN	meganuclease* and plant* meganuclease* and crop* LAGLIDADG and plant* "homing endonuclease*" and plant* "homing endonuclease*" and crop* (I-SceI or I-CreI or I-DmoI) and plant* (I-SceI or I-CreI or I-DmoI) and crop* "rare cutting endonuclease" and (plant* or crop*)
TALEN	tale and nuclease* talen taln "Transcription activator–like effectors"
targeted mutagenesis in general	"targeted mutagenesis" and plant* "gene targeting" and plant* "targeted gene repair" and plant* "mismatch repair" and plant*
NEGATIVE SEGREGANTS	
RdDM	"rna-dependent dna methylation" "rna-directed dna methylation" "transcriptional gene silencing" and plant* not post RdTS and silencing RdDM
REVERSE BREEDING	"reverse breeding" “crossover control” AND breeding AND plant*
EARLY FLOWERING	"early flowering" and transgene "early flowering" and transformation GM and "early flowering" not transgene not transformation MADS-box gene accelerated flowering
VARIANTS OF PLANT TRANSFORMATION	
CISGENESIS - INTRAGENESIS	cisgenesis or cisgenic or cisgene* intragenesis or intragenetic or intragene "all native" AND plant* AND transformation

GRAFTING ON GM ROOTSTOCK	"gm rootstock*" "transgenic rootstock*" rootstock* and transgenic Transgrafting OR transgrafted

5.1.2 Literature data analysis

Both obtained review papers and research papers were categorised according to:

- Year of publication;

- Country (based on the address of the author(s)); all addresses were considered, in order not to loose information, due to the low number of publications;

- Institutions from academy or industry (based on the address of the author(s)).

- The 5-year impact factor of the journal, according to the Journal Citation Reports (JCR) – data of 2012. The three papers with the highest impact factor were identified for each technique (more than three in case of equivalent value).

Research papers additionally were categorised according to:

- Plant on which the technique was used;

- Trait obtained through the application of the technique;

- For ZFN techniques: use of ZFN 1, 2 or 3 (see definitions in section 1.4.1); same for MGN and TALEN techniques

Data for all techniques were aggregated according to the year, the country and private/public distribution. No aggregation for plant and trait was performed, since not all techniques are applicable to the same plants and for the obtainment of the same traits. General conclusions were drawn on the overall results.

All publications collected were also screened manually to identify the reference to food/feed or environment safety issues and regulatory issues.

5.2 Field trials analysis for cisgenesis, intragenesis and grafting on GM rootstock

The applications for field trials submitted in the EU under Directive 2001/18/EC between October 2002 and December 2011 have been screened for the identification of plants obtained

through cisgenesis, intragenesis or grafting on GM rootstock. The database of the Institute for JRC-IHCP was used for the research:

http://ihcp.jrc.ec.europa.eu/facilities/Database_on_the_notification_for_GMO_releases.htm

The database contains all EU applications for field trials of plants carrying stably inserted genes and therefore also the products of cisgenesis, intragenesis and the GM rootstocks. The database contains the summary of the notifications of trials with GM plants which are fed into the system by the national competent authorities which receive them by applicants. Data in the database include: organism, type of genetic modification, period of release, purpose of the release, and additional data as required by the current legislation.

The criteria of search was based on the information provided by the applicants concerning the type of modification, genetic material inserted and the brief description of the method used for genetic modification. It is noted that the questionnaire used for the application is targeted on transgenic crops. Additionally, the quality and detail of the information provided is not homogenous between notifications. The type of modification is specified as insertion in all applications. Details of the inserted genetic material are varying and especially information on the intended function and the source of genes are sometimes missing. Concerning the method applied, usually only the method of delivery is specified. The methods used for selection are rarely reported.

Field trials databases of other countries were investigated for suitability for the search, like e.g. the databases of the US Department of Agriculture (http://www.nbiap.vt.edu/search-release-data.aspx) and the International Service for the Acquisition of Agri-biotech Applications (http://cera-gmc.org/index.php?action=gm_crop_database). However, the US databases do not provide the same detail of information on the inserted construct as the EU database. Only the name of the gene of interest and the marker gene are specified. Additionally, no application specifies the use of the plants as rootstocks. Therefore, these databases were regarded as not suitable to carry out a search for crops obtained by any NPBT.

5.3 Patent search on NPBTs

5.3.1 Patent databases and searches methods

Three public patent databases were selected for the search: WIPO (World Intellectual Property Organization), EPO (European Patent Office) and USPTO (United States Patent and Trademark Office). The patent search was finalised in August 2012. Patent applications are published 18

months after filing. That means that only patents filed by the beginning of 2011 are included in the findings. The methodology employed for the search is briefly explained below and described into more detail in Parisi *et al*. (Parisi *et al.*, 2012).

Results of the search include both patent applications and issued patents. The patent search was performed for the same list of NPBTs subject to literature search.

The search for patents registered by WIPO and EPO was performed through the function "advanced search" in the EPO website www.ep.espacenet.com, in which both WIPO and EPO databases can be selected for the search. Different keywords and combinations of keywords were used for the search in the full text of the patents. The same keywords were used for searching both in WIPO and EPO.

The function "classification search" of the same website has also been tested. Some European Classification (ECLA) codes were identified that could include patents of interest (i.e. category of enzymes, category of genetic engineering, category of gene silencing, etc.), but they revealed to be too general compared to the very specific search needed for the techniques selected and were abandoned.

The search for patents registered by the USPTO was performed through the USPTO website http://patft.uspto.gov. Both AppFT (patent applications) and PatFT (granted patents) databases were explored through the function "advanced search". In the query box, the same keywords used for the previous searches were inserted after the word "spec", which directs the search to the whole text of description of the patent.

Boolean operators (AND, OR, NOT) and Truncation wildcards, like the asterisk * for the search of words of different length, were employed in order to refine the search. Quotation marks were used to find words that must appear adjacent to each other (i.e. "zinc finger").

In some cases, searches for the inventor's name and applicant institutions were also performed with the aim of double checking the obtained results or in order to identify missing patents. Data retrieved from the literature search were taken into consideration for this search.

Applicants often patent their inventions in several patent offices. They might apply both in EPO and USPTO, or they might prosecute the international PCT application first (registered in WIPO) and decide to protect later in the EU (through EPO) or in the USA (through USPTO) or both. Therefore, duplicates or triplicates were frequently found by searching in the three databases and were eliminated. Each patent represents also all members of its patent family.

The list of keyword combinations employed in the patent search for the new techniques is presented in Table 5.2. Keywords that were discarded because of lack of results are not presented. Keywords used for the literature search were tested, but in many cases more specific

combinations were used in order to reduce the list of results. Patent descriptions are very detailed and include examples and references, therefore, simple keywords can be found in a large number of patents.

Tab 5.2 Keywords employed in the patent databases of EPO, USPTO and WIPO for the search of patents related to NPBTs

Technique	Keywords
TARGETED MUTAGENESIS	
ZFN	"zinc finger " AND nuclease* AND plant AND break "zinc finger" AND NHEJ
ODM	"chimeric oligonucleotide*" AND plant "chimeric mutational vectors" oligonucleotide* and plant* and mutation*
MGN	meganuclease* and (plant* or crop*) (I-SceI or I-CreI or I-DmoI) and (plant* or crop*)
TALEN	(tale or talen) and nuclease* "tal effector*" or "Transcription activator–like effectors"
NEGATIVE SEGREGANTS	
RdDM	transcriptional AND "gene silencing" AND TGS AND plant RdDM AND plant
REVERSE BREEDING	"reverse breeding" “crossover control” AND breeding AND plant*
VARIANTS OF PLANT TRANSFORMATION	
CISGENESIS - INTRAGENESIS	cisgenesis or cisgenic or cisgene* intragenesis or intragenetic or intragene "all native" AND plant* AND transformation
GRAFTING ON GM ROOTSTOCK	graft* AND rootstock* AND transgenic "transgenic rootstock*" "GM rootstock*"

5.3.2 Patent data analysis

The list of patents obtained for each technique through the keywords was manually screened in order to select patents describing the intentional use of the technique within the scope of plant breeding. Non-relevant patents were eliminated.

In case of patents referring to more than one technique (e.g. TALEN and ZFN techniques) the patent application was associated to the technique most relevantly represented.

Patents obtained were categorised according to:

- Priority date (date of first application);

- Country of assignee/s (as indicated in the patent application);

- Assignee from academy or industry;

- Claimed plant/s;

- Claimed trait/s obtained through the application of the technique.

- Geographic coverage of the patent families

Data for all techniques were aggregated according to the year, the country and academy/industry distribution. No aggregation for plant and trait was performed, since not all techniques are applicable to the same plants and for the obtainment of the same traits. General conclusions were drawn on the overall results.

All patents (and patent applications) collected with the method described above were grouped according to the assignee. The main assignees in terms of patent portfolio were identified in the whole field of NPBTs. In addition and in order to complete the information retrieved in patents about key organisations, potential products close to the market and licensing agreements, official Websites of the main institutions emerging from patent search have been analysed, investigating data on specific products or licensing agreements.

5.3.3 Analysis of patent citations and claims

For each patent collected, all associated cited patents were identified for the analysis of knowledge spillovers in NPBTs, as illustrated in Hall et al. (2001) and Johnson and Lybecker (2012).

Patents are usually cited (by the inventor or by the examiners of the patent office) when the invention they claim is the basis for the patent being analysed. Therefore, it is likely to be necessary that the applicant licenses it to be able to realise its invention and put it on the market. Accordingly to this principle, the key patents for each technique were identified as the patents cited by most other patents. This exercise was performed first by identifying the most cited patents within the group of patents identified per technique (findings described in section 5.3.1) to identify the key actors in the specific field of the NPBT analysed. Then, most cited patents outside the group of patents on the specific technique were analysed; they could be, e.g. patents not claiming application in plants or patents about one technology component in the process. Claims of identified patents were analysed to complement citation analysis.

In each patent, claims are structured in a hierarchal way, in which there are few independent claims (always the first one, sometimes some more) and many other dependent claims. Patent subjects can be processes (methods) or products, but some patents can claim a mixture of both,

which is usually the case for patents in the field of plant breeding techniques. The first claim of a patent (which is independent) can be considered as the dominant claim. Therefore, a classification of patents content between process and product were performed on the basis of the content of the dominant claim only, considering it as the main subject of the patent (even if not the only one) with scope of giving a general idea of the prevalent type of protection in patents on NPBTs.

5.4 Comparative analysis of plant breeding techniques

5.4.1 Case studies selection

The comparative analysis between plant breeding techniques was performed through case-studies in which a crop variety with a specific new trait is obtained through a NPBT or conventional breeding. The selected case studies are the following:

Case study 1: Wheat with improved bread making quality, obtained through either introgression breeding, transgenesis or cisgenesis

Case study 2: Dwarfing citrus, obtained through either conventional breeding or grafting on GM rootstock

In the choice of case-studies the first criterion was to include the most advanced NPBTs, i.e. techniques whose proof of concept was already obtained in crop plants and not just in model plants, like cisgenesis and grafting on GM rootstock. This requirement assures that the data we obtain are as close as possible to reality. The second criterion was to select case-studies in which it was possible to apply the two alternative techniques for the obtainment of the same trait (a conventional technique and a new technique). For this objective, we have given priority to the cases in which the same institution possessed data about the two techniques, in order to avoid too much variability in expenses' measure among the two techniques. Therefore we have chosen IAS-CSIC for cisgenesis and introgression breeding in wheat and IVIA for grafting on GM rootstock and conventional methods.

5.4.2 Data collection

All data for the comparative analysis (described in more details in the next section) were provided by the experts working in IAS and IVIA on the subjects of the case-studies. During the whole data collection, the experts were interviewed in person, by e-mail and by telephone.

5.4.3 Comparative analysis

The methodology chosen as most suitable for the comparative analysis of the alternative breeding techniques is the spreadsheet-based approach, similarly as employed by Dreher et al (2003) and Bagge and Lubberstedt (2008) in their studies. Like for them, in our case details in the calculation are fundamental to highlight the differences between the technical processes.

In particular, Dreher and colleagues from the CIMMYT (International Maize and Wheat Improvement Centre), in Mexico, established a case study to perform the comparative analysis between conventional and marker-assisted maize breeding (Dreher *et al.*, 2003). Therefore, they first established a well-defined breeding objective: the identification of plants carrying a specific allelic form of the gene *opaque2,* involved in aminoacids composition. Field operations and laboratory procedures required for achieving the breeding objective were identified and their costs were determined by using a spreadsheet-budgeting approach.

As reported before, our analysis investigates the costs related to the stages of development of the plant variety and of varietal release, including technical costs, labour costs and regulatory costs, and take also into consideration measures of time in the breeding phase.

The protocols and schemes of the techniques analysed in the case-studies were prepared with the help of the experts form IAS and IVIA. Additionally, the following data on costs were reported:

- "Technical" costs: refer to the physical inputs required for the breeding operations, including chemicals and consumable employed in the different analysis and procedures for obtaining the new plant variety (Brennan and Martin, 2007) through the specific technique
- Labour costs: related to salaries and associated costs of people required for performing all the breeding operations (Brennan and Martin, 2007)
- Regulatory costs: refer to the costs for the introduction of a plant variety into the market.

As described in section 2.2.4, regulatory costs differ between the products of transgenesis, which are included in the scope of the GMO Directive 2001/18/EC, and the products of conventional breeding, excluded from that Directive. Therefore, for transgenesis we need to calculate the approximate value of the regulatory process to bring a GM plant to the market. For conventional breeding products, the regulatory costs would only include the variety registration. For the case of a NPBT (cisgenesis and grafting on GM rootstock), two hypothetical scenarios are evaluated: i) the product is considered as a GMO, so the risk assessment costs need to be undertaken and ii)

the product is excluded from the GMO Directive, so only costs for varietal registration are considered.

In addition to cost comparison among technique, time requirements are also an important factor to consider in the analysis (Morris *et al.*, 2003). According to Pandey and Rajatasereekul (1999), a longer breeding cycle also implies an economic cost related to the delay in realising the variety and consequently in the benefits of commercialisation. Therefore, the average duration of the application of each technique will also be compared.

The research costs related to the development of the technology and the discovery of the genes implied are not included in the analysis. In the comparison it is assumed that all the knowledge is already available. Due to the novelty of the NPBTs, the study does not include adoption stage, since no data are available for the time being. Costs related to the establishment of the breeding stations and the laboratories (capital costs) are not included in the analysis. It is assumed that a breeding station generally possess all tools for creating a new plant variety with the three techniques, so that they can be considered as equal for the three cases.

Therefore, the starting point of the analysis is a breeder who possesses all the knowledge/know-how to develop the plant with the new trait and disposes of an equipped laboratory. The ending point is the commercial registration of the new plant variety.

5.5 Analysis of Freedom to Operate in NPBTs

In plant breeding it is sometimes necessary to make use of tools that are protected by a patent (or more than one) and this is more likely when using molecular tools. According to Art. 30 of the WTO's TRIPS agreement (http://www.wto.org/english/tratop_e/trips_e/trips_e.htm), those tools are subject to exemption to the rights conferred by patents when used for research purposes only. In the case-studies, however, it is considered that the final variety is produced for commercialisation. Therefore, licensing revenues of all patented tools employed in the process have to be included in the costs.

The analysis of FTO was performed for the case-study of dwarfing GM citrus rootstock, based on the methodology described by Kowalski et al (2002). However, the objective of this analysis was not to provide IVIA with specific data on patent infringements before commercialisation (like professionals on FTO do), but rather to give an illustrated example of the big amounts of patented methods and products involved in a biotechnological plant breeding process. Therefore, the analysis performed included the phase of product deconstruction and a general patent search to identify potential patents associated to each steps.

Each patent was analysed by reading the specifications and claims. Claim scope interpretations were based only on literal interpretations. For each patent identified in relation with the protocol for GM rootstock case-study, all members of the INPADOC family were listed with their filing dates in order to define patents' geographic coverage. Expiry dates were calculated based on standard patent expiration schedules, following the rules established in each country, in most cases calculated at 20 years from their earliest claimed priority date.

5.6 Experts' opinion

In the course of this study on NPBTs, several experts in the field of NPBT, both from academy and industry, have been approached, in particular during a Workshop on "New plant breeding techniques: Adoption and economic impact", held on 27th and 28th May 2010 IPTS, a training session on the "Application of new plant breeding techniques" at Wageningen UR, Plant Breeding (NL) and a few meetings with the experts performing the evaluation "Changes in the genome of crops caused by the application of new plant breeding techniques" (Glandorf, 2011).

Thereby, beside the information retrieved from literature and patent search, additional information and opinions have been collected on NPBTs, in particular on drivers and constraints of NPBTs, on safety and regulatory issues and on plant breeding and licensing costs.

Chapter 6. Results: Research landscape on NPBTs

6.1 Introduction

This chapter is dedicated to the analysis of the research landscape in NPBTs and in particular to the information extracted from the results of a search in the scientific literature. The objective was to evaluate the development of research activities and to identify the leading countries and institutions in the field of NPBTs. The description of the methodology followed is provided in section 5.1 and the whole list of papers identified per each technique is provided in Annex I.

The following sections will present an overview of the information obtained per technique in terms of number and dates of publications, country of origin of the authors and public or private sector. The results also allow comparison of the research stages of each technique, by differentiating for example between those still applied only to model plants and traits and/or those already being applied to agriculturally relevant crops and traits. The publications with higher impact factor were also identified to determine the most relevant authors in the field of NPBTs. Also, the information retrieved from the EU database of field trials is illustrated.

In addition to these data, more information was extracted from the scientific literature, in particular to identify safety and regulatory issues related to NPBTs, to determine the factors that favour or hinder the adoption of these techniques by the plant breeding sector.

6.2 Research in NPBTs

The information about scientific publications in the field of NPBTs is presented in the next sections first technique by technique and finally as aggregated data for the whole field. The data for "targeted mutagenesis" techniques has been aggregated and analysed separately due to the high homogeneity of this group of techniques, both from the point of view of scope and of technical procedure.

6.2.1 Targeted mutagenesis: ODM, ZFN, MGN, TALEN

Scientific literature on targeted mutagenesis (see definition in section 1.4.1) is particularly abundant in the field of medical biotechnology. There are several human diseases that are caused by point mutations in fundamental genes, both hereditary diseases and new diseases. Therefore, it is of great interest to develop methods of targeted modification of single nucleotides with the aim to correct these genetic defects (Parekh-Olmedo and Kmiec, 2007;

Urnov *et al.*, 2010; Miller *et al.*, 2011; Silva *et al.*, 2011). Targeted gene therapies have emerged as potential strategies for treatment of such diseases. The scientific literature of targeted mutagenesis techniques reveals also many applications to animal cells, in particular of zebrafish (Takahashi and Dawid, 2005; Doyon *et al.*, 2008; Sander *et al.*, 2011a), mice (Gouble *et al.*, 2006; Aarts and Riele, 2011) and rats (Tesson *et al.*, 2011). In many cases the scope of the experiments with these techniques in animals is to obtain proof of concept before to test the method in human cells.

The scope of this study is the use of the techniques of targeted mutagenesis in plants. Since the technical process is the same, the search also included publications describing targeted gene insertion in plants for ZFN, MGN and TALEN techniques. The findings of the literature search about plants will be illustrated in the next paragraphs per single technique and finally as aggregated data. Due to the novelty of all four techniques and therefore the reduced number of publications compared with older techniques, both model plants and crop plants have been included in the scope of the search. The term "model plants" refer to those plants (like *Arabidopsis* and tobacco) that have been extensively studied from a genetic point of view and are mainly use to understand particular biological processes and therefore represent a preliminary use of a technique, before its application in plants of interest (like crop plants). Additionally, a distinction is made between targeted modification or insertion of marker genes like the genes encoding GFP (Green Fluorescent Protein) and GUS (beta-glucuronidase) and antibiotic resistance genes, and the use of the technique with genes that produce a new trait of potential interest in agriculture. These two last parameters (use in crop plants and for traits of agronomic interest) can also be considered a preliminary measure of how plant breeding techniques are advanced, in terms of closeness to commercial applications.

Oligonucleotide directed mutagenesis (ODM)

Ten research papers and 15 review papers have been identified on the use of ODM in plants. From the distribution of these publications over time (Figure 6.1), it can be observed that most publications were concentrated between 1999 and 2006. No more research papers have been produced afterwards, only two reviews. According to the geographical distribution of authors shown in Table 6.1, North America (especially US) produced the highest number of publications, followed closely by the EU. By analysing to which sector the Institutions of origin of authorships belong, what emerges is that publications from the academic sectors are clearly prevailing for ODM (21 papers). Only three papers have been produced in the industry sector and one by authors belonging to both sectors (joint authorship).

Additionally, the most active institutions for ODM have been identified by analysing the first three research papers with the highest 5-year impact factor (as shown in Table 6.2). The company Pioneer Hi Bred International and the academic Boyce Thompson Institute for Plant Research of Cornell University, both from the US, have produced the most relevant papers on ODM

Finally, each research paper has been analysed to identify in which plants the technique has been used and for the obtainment of which trait and the results are shown in Table 6.3. Some examples of applications in crop plants are available in literature for ODM: the technique has been used in rice and oilseed rape to mutate the gene *SuRA* econding ALS (acetolactate synthase) and in maize to mutate the gene encoding AHAS (acetohydroxyacid synthase), in both cases to obtain herbicide tolerant plants. Papers also report the use of ODM to mutate the *SuRA* gene in the model plant tobacco, and to introduce mutations in marker genes like antibiotic resistance genes and *gfp* in several crop plants (maize, banana, wheat and oilseed rape) and model plants (*Arabidopsis* and tobacco).

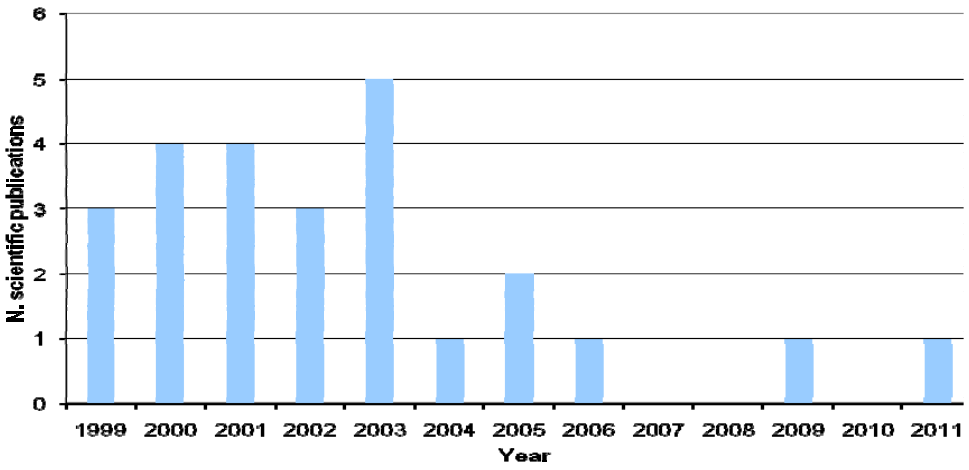

Figure 6.1 Development over time of scientific publications on ODM in plants.

Table 6.1 Geographical distribution of scientific publications on ODM in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**9**
Germany	6
Belgium	2
UK	1
North America	**13**
USA	12
Canada	1
Asia	**2**
Japan	2
Other countries	**2**
Australia	1
Switzerland	1
UN*	1

* UN = United Nations (referring to a publication by FAO and IAEA)

Table 6.2 First three papers on ODM in plants with the higest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Zhu *et al.* (2000)	Nature Biotechnology	28.161	Pioneer Hi-Bred International, Inc (US)
Beetham *et al.* (1999)	PNAS	10.472	Boyce Thompson Institute for Plant Research, Cornell University (US)
Zhu *et al.* (1999)	PNAS	10.472	Pioneer Hi-Bred International, Inc (US)

Table 6.3 Plants and traits for which ODM is used according to research papers identified.

Plant	Traits	N. publications
Tobacco	HT, antibiotic res., reporter gene	4
Arabidopsis	antibiotic res.	1
Maize	HT, antibiotic res.	3
Oilseed rape	HT, antibiotic res.	2
Rice	HT	1
Wheat	reporter gene	1
Banana	antibiotic res.	1

Zinc finger nuclease (ZFN) techniques (ZFN 1,2,3)

By including all three uses of ZFN techniques (ZFN 1,2,3) in the search, 16 research papers and 16 review papers have been identified. According to the time distribution of ZFN publications illustrated in Figure 6.2 almost all publications are concentrated in the last seven years, confirming the novelty of the technique, especially for its application in plants. By analysing the distributions of all authors involved and the country of their institutions (Table 6.4), US-based authors have a clear predominant role, while EU-based authors only produced 4 publications. Most institutions involved in publishing about ZFN techniques in plants come from the academic sector (23 papers). The industry sector produced 4 papers on ZFN in plants, while 5 are from joint authorship.

The most relevant papers in terms of impact factor (Table 6.5) have been all produced by US institutions, including three companies in the first positions (Dow AgroSciences, Sangamo BioSciences and Phytodyne, Inc.) and several academic institutions like Iowa State University.

Regarding the information extracted on plants and traits (Table 6.6), ZFN-1 technique has been used in the model plants tobacco and *Arabidopsis*, in the crop plant soybean and in the ornamental plant petunia. In tobacco it was used to obtain herbicide tolerance through the mutation of genes encoding ALS gene and for mutating reporter genes encoding GUS and GFP. ZFN-1 technique was used in *Arabidopsis* for the mutation of genes involved in abiotic stress resistance, of endogenous genes for research purposes and of marker genes. Finally, ZFN-1 technique was used in soybean and petunia for mutation of marker genes or endogenous genes with research scope mainly. For the ZFN-2 technique, research papers report its use on the model plants tobacco and *Arabidopsis* for the mutation of marker genes. ZFN-3 was used for the integration of the gene enconding PAT (phosphinothricin phosphotransferase) that confers herbicide tolerance into tobacco and maize and at the same time to obtain insertional mutagenesis of the gene encoding IPK (inositol-1,3,4,5,6-pentakisphosphate 2-kinase) for obtaining reduced phytate content. An additional publication describes the use of ZFN in tobacco for the removal of a transgene, which is outside the definition of ZFN 1,2,3 but follows the same technical procedure and has also a potential for agricultural applications.

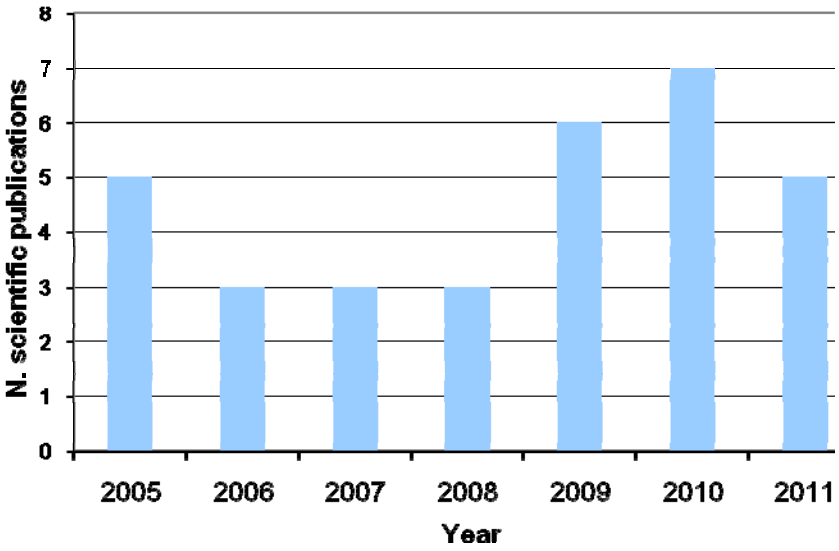

Figure 6.2 Development over time of scientific publications on ZFN techniques in plants.

Table 6.4 Geographical distribution of scientific publications on ZFN techniques in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**4**
Germany	2
Netherlands	1
France	1
North America	**24**
USA	24
Asia	**4**
Japan	2
Korea	1
India	1
Other countries	**5**
Israel	3
Switzerland	2

Table 6.5 First three papers on ZFN techniques in plants wih the highest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Shukla *et al.* (2009)	Nature	36.235	Dow AgroSciences (US) Sangamo BioSciences (US)
Townsend *et al.* (2009)	Nature	36.235	Phytodyne, Inc. (US) Iowa State University (US)
Sander *et al.* (2011)	Nature Methods	20.454	Massachusetts General Hospital (US) Harvard Medical School (US) University of Minnesota (US) Yale University School of Medicine (US) Iowa State University (US) Broad Institute (US)

Table 6.6 Plants and traits for which ZFN techniques are used according to literature

Plant	Traits	Techniques	N. publications
Tobacco	HT, reporter genes (including removal)	ZFN 1,2,3	8
Arabidopsis	reporter genes, abiotic stress, alcohol res.	ZFN 1,2	6
Soybean	reporter genes	ZFN 1	2
Maize	HT, phytate reduction	ZFN 3	1
Petunia	reporter gene	ZFN 1	1

Meganuclease (MGN) techniques (MGN1,2,3)

Compared to the techniques previously described, fewer publications have been identified for targeted mutagenesis or integration making use of MGNs, although the homing nucleases are already known since long time. Eleven publications (10 research papers and one review) have been indentified in scientific literature about the use of MGNs for the obtaining of double strand breaks in the genome of target plants and consequently either site-specific mutations or insertions. However, it is important to highlight that MGNs already possess sequence specificity towards a restriction site. Most papers identified describe a previous step in which target plants are transformed with marker genes which possess a recognition site for MGNs. Afterwards, genes encoding MGNs are delivered to be expressed and to cut the site previously inserted. Only in one paper (Gao *et al.*, 2010), MGNs are artificially modified to direct their specificity to a new target endogenous gene.

According to the distribution over time shown in Figure 6.3, first publication related to the use of MGNs for targeted mutation in plants appeared in 1998 (Salomon and Puchta, 1998). According to Table 6.7, publications are distributed between EU and US institutions. Six papers have been

published by academy, four by industry and one by join authorship. Analysing the three papers with highest impact factor (Table 6.8) the most relevant institutions in the field are the University of Texas in the US and the IPK (lnstitut fur Pflanzengenetik und Kulturpflanzenforschung) in Germany.

According to literature findings (Table 6.9), MGN 1 was used in *Arabidopsis* for research purposes, such as mutation of endogenous genes (involved in photosyntesis) or introduction of marker genes. In tobacco MGN 1 technique was applied also for mutation of marker genes and MGN 3 for site specific integration of a gene (PAT) for herbicide tolerance. According to literature, the only crop plant in which MGN was used so far is maize. In maize, MGN 1 technique was applied by artificially modifying the DNA recognition domain of a MGN and direct its specificity to an endogenous locus related to leaf structure. MGN 2 technique was used in maize to modify a marker gene and MGN 3 technique to site-specifically integrate marker genes.

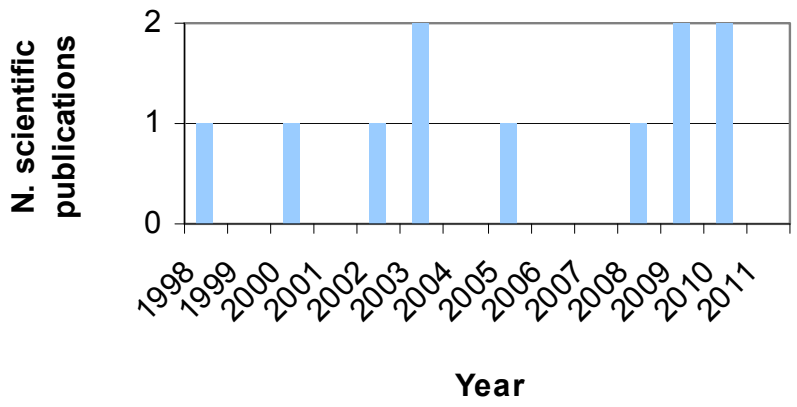

Figure 6.3 Development over time of scientific publications on MGN techniques in plants.

Table 6.7 Geographical distribution of scientific publications on MGN techniques in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**6**
Germany	4
France	1
Belgium	1
North America	**5**
USA	5

Table 6.8 First three papers on MGN techniques in plants wih the highest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Kwon *et al.* (2010)	PNAS	10.472	University of Texas (US)
Kirik *et al.* (2000)	EMBO Journal	8.833	lnstitut fur Pflanzengenetik und Kulturpflanzenforschung (IPK) (DE)
Salomon and Puchta (1998)	EMBO Journal	8.833	lnstitut fur Pflanzengenetik und Kulturpflanzenforschung (IPK) (DE)

Table 6.9 Plants and traits for which MGN techniques are used according to literature

Plant	Traits	Techniques	N. publications
Tobacco	Loss of marker gene function, Antibiotic res.	MGN 1,3	5
Arabidopsis	Photosynthetic activity, loss of marker gene function	MGN 1	3
Maize	Leaf structure, antibiotic res. and HT	MGN 1,2,3	3

Transcription Activator-Like Effector Nuclease (TALEN) techniques

Literature search on TALEN confirmed the novelty of the technique in plants, since the few identified publications (5 research papers and 2 review papers) are concentrated in the last 2 years, as illustrated in Figure 6.4. The findings include both publications describing the use of TALEN for site-directed mutagenesis and the use of modified TAL-effectors for recognising a desired promoter sequence and activate the expression of the corresponding gene. It is considered that this specificity is the premise for the use of TALEN for producing a DSB and consequently a mutation.

According to Table 6.10, the authors of these publications are distributed among Germany, the US, China and Saudi Arabia, mostly from academy: 5 papers against one from industry and one from joint authorship. According to Table 6.11 the most relevant institutions in literature in the field of TALEN techniques in plants are from academy and in particular form Saudi Arabia (King Abdullah University of Science and Technology), Germany (University of Munich and Martin-Luther-University), from the US (University of Minnesota, Iowa State University and University of Minnesota Rochester) and from China (University of Electronic Science and Technology of China).

Plant and traits (Table 6.12) have been divided also according to the use of TAL-effectors, as specified in the introductory paragraph: for targeted mutagenesis through a nuclease or for targeted gene activation. For the first use described, according to literature only model plants (*Arabidopsis* and tobacco) have been employed: for the mutation of the gene encoding ADH (Alcoohol Dehidrogenase) in *Arabidopsis* and for the mutation of a marker gene in tobacco. Modified TAL-effectors were used to activate the promoter of a marker gene in tobacco and promoters of endogenous genes in *Arabidopsis*, tomato and pepper. According to a research paper published in 2012 (and therefore outside the literature results) (Li *et al.*, 2012), TALEN techniques were also used in rice for conferring bacterial rice blight resistance by targeting the gene *Os11N3*.

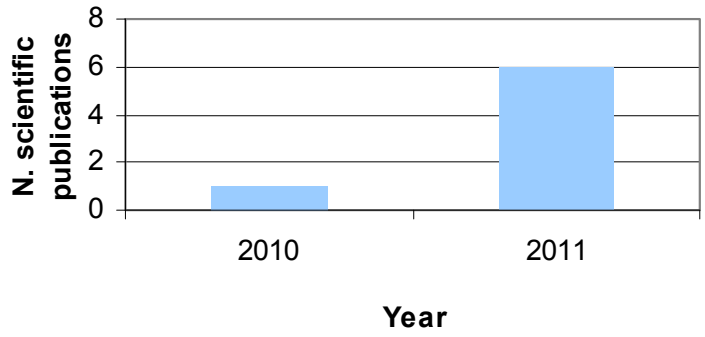

Figure 6.4 Development over time of scientific publications on TALEN techniques in plants.

Table 6.10 Geographical distribution of scientific publications on TALEN techniques in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**3**
Germany	3
North America	**2**
USA	2
Asia	**3**
Saudi Arabia	2
China	1

Table 6.11 First four papers on TALEN techniques in plants wih the highest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Mahfouz *et al.* (2011)	PNAS	10.472	King Abdullah University of Science and Technology (SA)
Morbitzer *et al.* (2010)	PNAS	10.472	University of Munich (LMU) (DE) Martin-Luther-University (DE)
Cermak *et al.* (2011)	Nucleic Acids Research	7.417	University of Minnesota (US) Iowa State University (US) University of Electronic Science and Technology of China (CN) University of Minnesota Rochester (US)
Morbitzer *et al.* (2011)	Nucleic Acids Research	7.417	University of Munich (LMU) (DE) Martin-Luther-University (DE)

Table 6.12 Plants and traits for which TALEN techniques are used according to literature

Plant	Traits	Techniques	N. publications
Arabidopsis	Mutation of ADH1 gene	TALEN 1	1
Tobacco	Marker gene mutation	TALEN 1	1
Tobacco	Reporter gene activation	TALE-mediated activation	2
Arabidopsis	Endogenous gene activation (leaf structure)		1
Tomato	Endogenous gene activation		1
Pepper	Endogenous gene activation (plant defence)		1

Targeted mutagenesis – aggregated data

Due to the very similar effect on the plant, the literature findings about the four targeted mutagenesis techniques analysed in this section have been aggregated. Publications about targeted mutagenesis and targeted integration in plants are concentrated in the last decade and a growing trend can be observed (Figure 6.5). According to Table 6.13, representing the geographical distribution of authorship, US-based authors play a prominent role in publishing about targeted mutagenesis and integration, followed by the EU. Most authors are from academic institutions (56 papers), while only 12 papers have been published by industry and 8 by joint authorship.

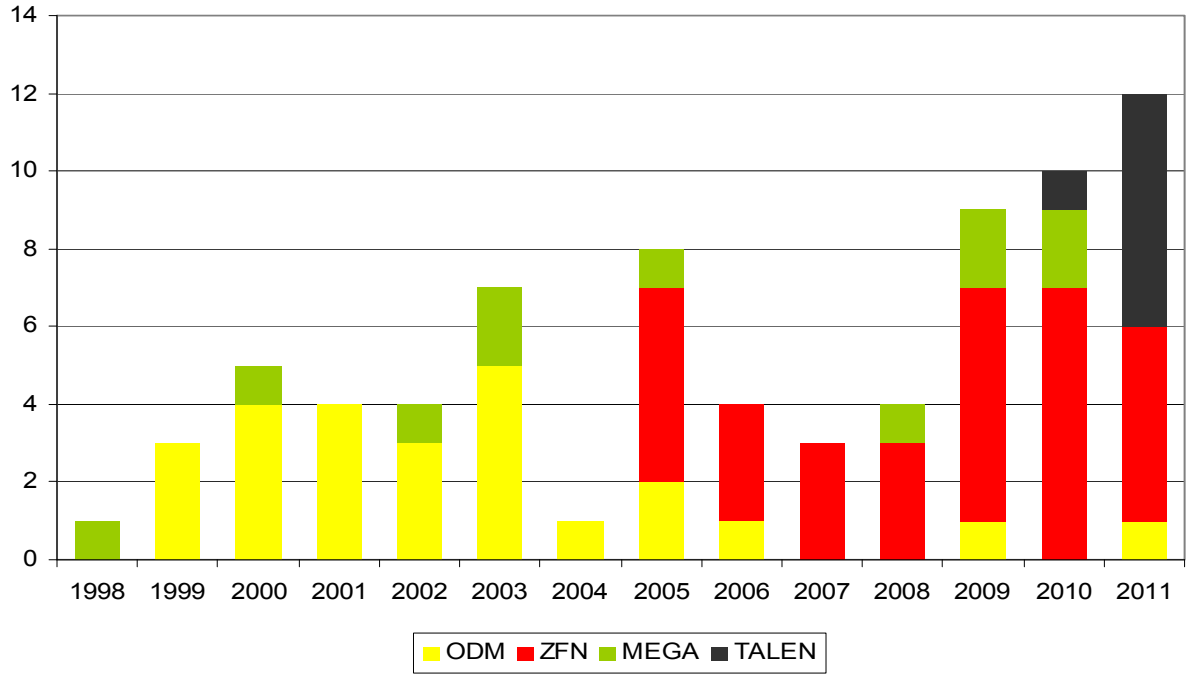

Figure 6.5 Development over time of scientific publications on targeted mutagenesis and insertion in plants.

Table 6.13 Geographical distribution of scientific publications on targeted mutagenesis and insertion in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**23**
Germany	16
Belgium	3
France	2
Netherlands	1
UK	1
North America	**44**
USA	43
Canada	1
Asia	**9**
Japan	4
Saudi Arabia	2
Korea	1
China	1
India	1
Other countries	**8**
Switzerland	3
Israel	3
Australia	1
UN	1

6.2.2 Techniques resulting in "Negative segregants": Reverse breeding, RdDM, Early flowering

RNA-dependent DNA methylation (RdDM)

Hundreds of publications have been already written about the mechanism of RdDM in plants. Among them, 40 (24 research papers and 16 review papers) have been identified on the intentional induction of this mechanism through the expression of a new sequence, which is then processed by the cellular system to produce dsRNA and guide the silencing of a target gene. In most cases though, the target gene for epigenetic silencing is a transgene (usually a marker gene) previously introduced into the plant genome. Only few research papers (eight) describe the use of RdDM for silencing of endogenous genes, but, according to literature, the silencing is less efficient compared to the silencing of transgenes. This might indicate that the technique is not yet advanced enough for a commercial use in plants.

According to Figure 6.6 the 40 publications indentified on RdDM induced in plants are concentrated in the last decade. EU-based authors are clearly dominating in terms of number of publications produced on RdDM (Table 6.14). They mainly belong to academy (35 papers), while three papers belong to industry and two to joint institutions. The institutions that published the three papers with highest impact factor are all academic (Table 6.15), three from Austria (Austrian Academy of Sciences, University of Veterinary Medicine and Boku University Vienna), one from the Netherlands (BioCentrum Amsterdam, Vrije Universiteit) and one from the UK (University of Cambridge).

According to the information reported in Table 6.16 on plants and trait, papers retrieved about induced RdDM describe its use in model plants, like tobacco and *Arabidopsis*, and for targeting of model genes (*nptII* and *gfp*). A few publications report the application of RdDM for the regulation of relevant genes in crop plants such as maize (male sterility), potato (granule-bound starch synthase gene), carrots (*carrot-leafy cotyledon 1, C-LEC1*, an embryo-specific transcription factor), tomato (genes involved in ripening), rice (*OsRac* genes) or in ornamentals (flower morphology and pigmentation).

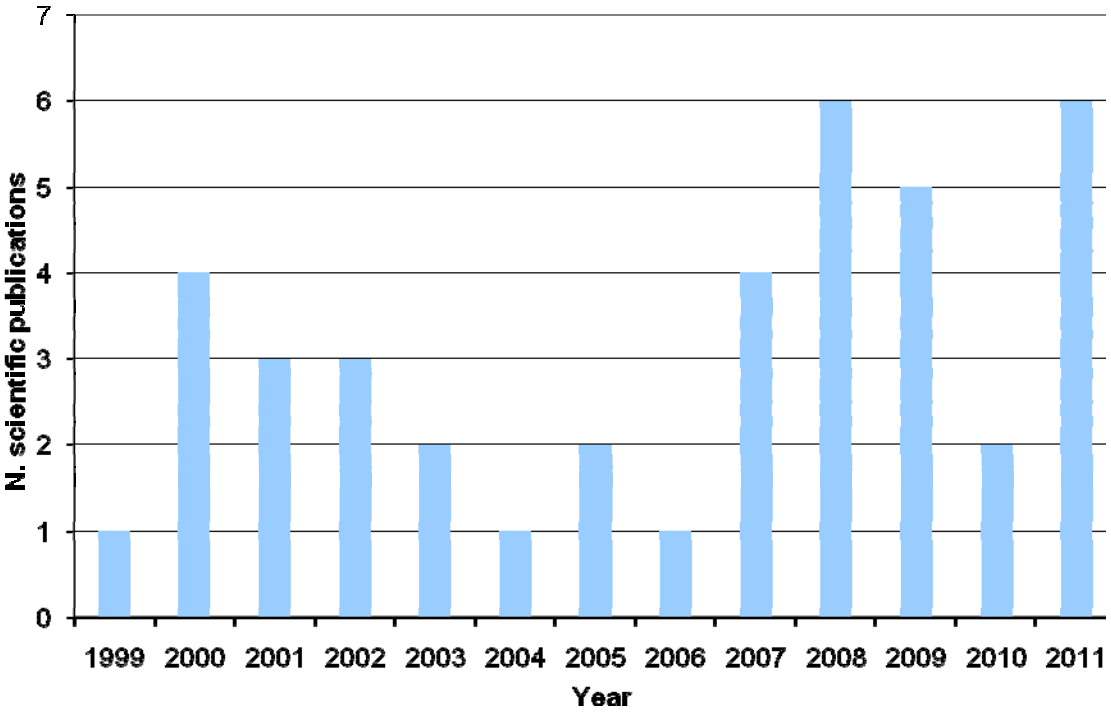

Figure 6.6 Development over time of scientific publications on RdDM in plants.

Table 6.14 Geographical distribution of scientific publications on RdDM in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**29**
Austria	12
Netherlands	4
UK	3
Germany	3
France	3
Czech Republic	2
Italy	1
Belgium	1
North America	**4**
USA	4
Asia	**9**
Japan	8
China	1
South America	**1**
Brazil	1
Other countries	**4**
Australia	2
Russia	2

Table 6.15 First three papers on RdDM in plants wih the highest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Kanno *et al.* (2008)	Nature Genetics	33.096	Austrian Academy of Sciences (AT) University of Veterinary Medicine (AT) Boku University Vienna (AT)
Sijen *et al.* (2001)	Current Biology	10.881	BioCentrum Amsterdam, Vrije Universiteit (NL)
Melnyk *et al.* (2011)	Current Biology	10.881	University of Cambridge (UK)

Table 6.16 Plants and traits for which RdDM is used according to literature.

Plant	Traits	N. publications
Arabidopsis	Silencing of antibiotic res. (transgene), reporter genes (transgenes)	10
Tobacco	Silencing of antibiotic res. (transgene), reporter genes (transgenes)	6
Rice	Silencing of reporter genes (transgenes), genes OsRac (endogenous genes)	3
Petunia	Altered flower morphology and pigmentation (endogenous genes)	3
Maize	Male sterility (endogenous gene)	1
Potato	Starch content (endogenous gene)	1
Tomato	No ripening (endogenous gene)	1
Carrot	Silencing of genes for embryo development (endogenous genes)	1

Reverse Breeding

Very few publications have been produced for the technique of reverse breeding to date; only four review papers have been identified, written between 2007 and 2010, and they do not refer to specific crops. One research paper was published in 2012 about the application of reverse breeding in *Arabidopsis* (Wijnker *et al.*, 2012). These data indicate that the technique is still in a preliminary phase of development. Table 6.17 illustrates the detailed information about the mentioned publications, including the one of 2012. It can be observed that Wageningen University from the Netherlands is present in authorship of almost all publications about this topic.

Table 6.17 Scientific publications identified about the use of reverse breeding.

Publication	Year	Country	Sector	Institution
Lammerts Van Bueren et al., 2007	2007	NL	academy	Wageningen University
		NL	academy	Louis Bolk Institute
		NL	academy	University of Amsterdam
Wijnker and de Jong, 2008	2008	NL	academy	Wageningen University
Dirks et al., 2009	2009	NL	industry	Rijk Zwaan Breeding BV
		AT	academy	Vienna University
		US	academy	Pennsylvania State University
		CN	academy	Fudan University
		NL	academy	Wageningen University
Chan, 2010	2010	US	academy	University of California
Wijnker et al., 2012	2012	NL	academy	Wageningen University
		NL	industry	Rijk Zwaan Breeding BV
		MY	academy	Universiti Putra Malaysia
		US	academy	University of California

Accelerated breeding through induction of early flowering

Research on the mechanism related to flowering time is being performed since long time. The first papers on the induction of early flowering in a plant through transformation were published in 1997 (Kang *et al.*, 1997; Kania *et al.*, 1997). *Arabidopsis* and tobacco model plants were transformed with genes (*FPF1* from *Arabidopsis* and *OsMADS7/8* from rice) that induced early flowering when over-expressed under a strong promoter. Since then, many more publications have been produced about the transformation of plants to obtain early flowering. Though literature search, 82 publications (77 research papers and 5 review papers) have been identified on this topic. According to their time distribution (Figure 6.7) they seem to have reached a peak of number of publications in 2007. However, in these publications it is not specified the employment of early flowering technique as a mean to accelerate breeding, with the intention of finally selecting only non-transgenic plants, as described in the definition of this new technique in section 1.4.3. Therefore, no further analysis has been performed on the publications identified.

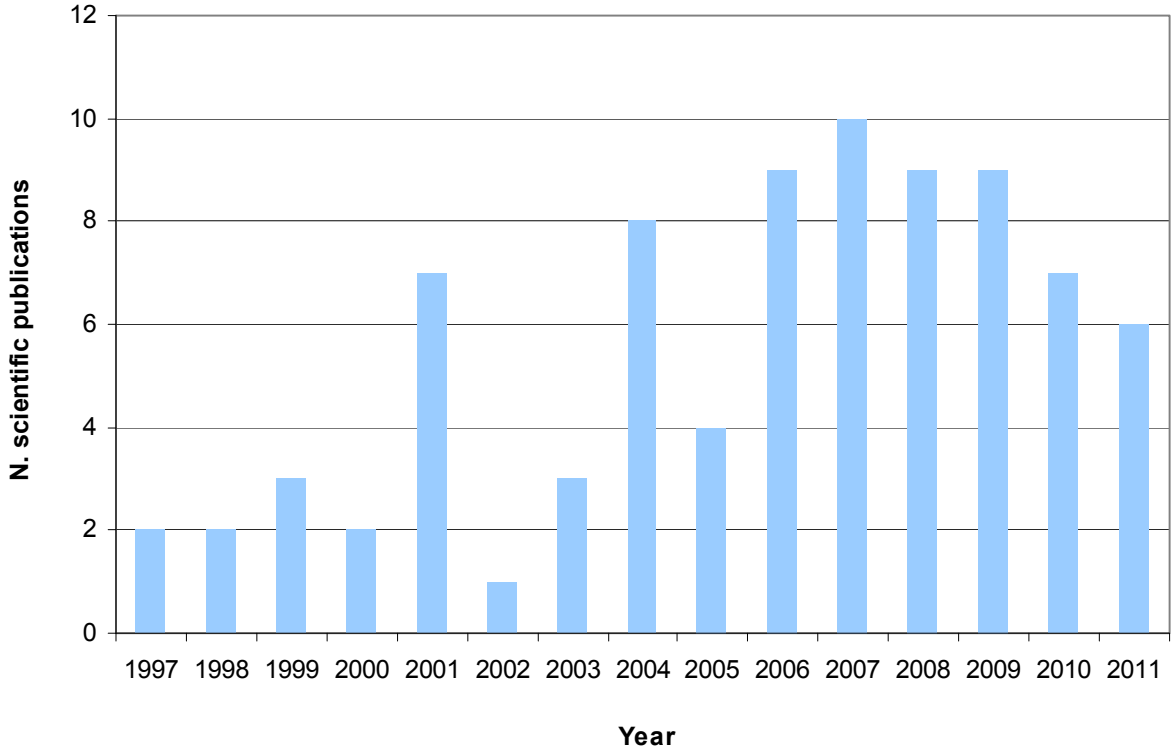

Figure 6.7 Development over time of scientific publications on induced early flowering.

6.2.3 Variants of plant transformation techniques: Cisgenesis, Intragenesis and Grafting non-GM varieties onto GM rootstock

Cisgenesis and Intragenesis

Cisgenesis and Intragenesis techniques were grouped together in the same search, since their definitions are often overlapping in scientific literature, especially in the first papers on the topic. Therefore, literature search for this group of techniques was based in the principle of transforming a plant with DNA sequences from a cross-compatible source. Following this scope, 16 research papers and 27 review papers were identified. Publications using the techniques of cisgenesis and intragenesis are produced since 2003 and seem to be still in a growing phase (Figure 6.8). Table 6.18 shows that EU-based authors are the most productive in terms of number of publications, and in particular authors from The Netherlands. US-based authors follow. Most authors in cisgenesis and intragenesis come from academic institutions: 30 papers against 9 from industry and 4 from joint institutions. According to Table 6.19 the institutions producing the publications with the highest impact factor on cisgenesis/intragenesis are the University of Bologna, together with the Swiss Federal Institute of Technology and the company

J.R. Simplot. However, it must be highlighted that Wageningen University does not emerge in this analysis since it has produced mostly review papers on cisgenic potatoes and apples, but they are considered as the parents of cisgenesis techniques and deserve to be considered in the top institutions for this technique.

According to literature information (Table 6.20), with the exception of one paper on intragenesis in the model plant tobacco for the integration of genes coding for restriction endonucleases (for research purposes), all the other publications on cisgenesis or intragenesis relate to plants with commercial interest like crop plants or trees: potato, apple, melon, grapevine, alfalfa and poplar. Traits introduced into potato include fungal resistance, black spot bruise tolerance and low level of acrylamide production. The technique is used in apple, melon and grapevine for obtaining fungal resistance. It is used in alfalfa to improve the lignin content of forage and in poplar to modify growth and wood properties. According to a research paper published in 2012 (and therefore outside the literature results) (Holme *et al.*, 2012), cisgenesis was also used in barley for the improvement of phytase activity.

Table 6.21 shows the findings about field trials notified in the EU and registered in the JRC-IHCP database for the cultivation of cisgenic and intragenic plants. In the database, five field trials of late blight-resistant potato obtained by the insertion of a gene derived from a wild relative were identified, together with one field trial on cisgenic apples transformed with a gene for apple scab resistance and one field trial on cisgenic barley with improved phytase activity. In all of them it is specified that the genes were delivered together with its own promoter and terminator and that the obtained plant does not carry any marker gene. In some of them it is specified that the T-DNA borders of *Agrobacterium* are still present in the final plant. Therefore, the stricter definition of cisgenesis (with absence of those borders) is not followed. In the database also four field trials were identified on potatoes with reduced amylose content (for starch production) that could be classified as intragenesis on the basis of the information provided on the genetic modification (for gene silencing).

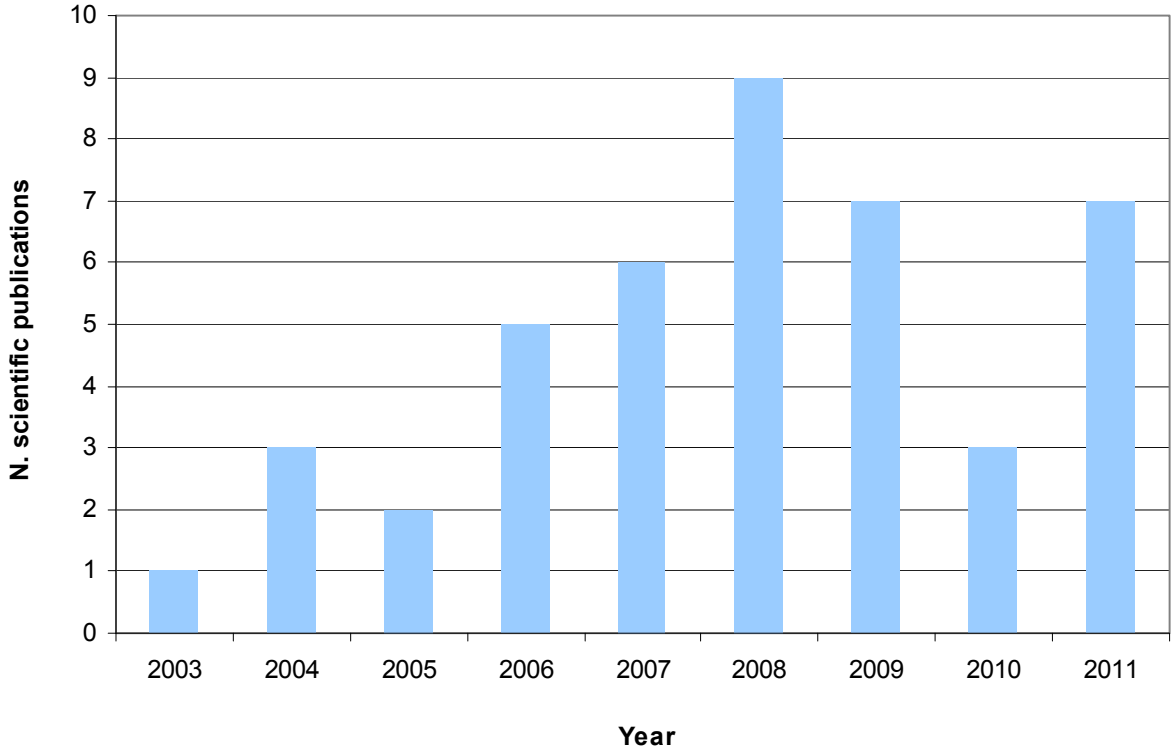

Figure 6.8 Development over time of scientific publications on cisgenesis and intragenesis in plants.

Table 6.18 Geographical distribution of scientific publications on cisgenesis and intragenesis in plants. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**26**
Netherlands	17
Italy	4
UK	3
Germany	2
North America	**15**
USA	15
Asia	**3**
India	2
Bangladesh	1
South America	**1**
Chile	1
Other countries	**2**
Switzerland	4
New Zealand	2
Norway	2
Australia	1
Israel	1

Table 6.19 First three papers on cisgenesis and intragenesis in plants wih the highest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Belfanti *et al.* (2004)	PNAS	10.472	University of Bologna (IT) Swiss Federal Institute of Technology (CH)
Rommens *et al.* (2004)	Plant Physiology	7.054	J.R. Simplot Company (US)
Rommens *et al.* (2005)	Plant Physiology	7.054	J.R. Simplot Company (US)

Table 6.20 Plants and traits for which cisgenesis and intragenesis are used according to literature.

Plant	Traits	N. publications
Tobacco	reporter genes	1
Apple	fungal res., reporter genes	6
Potato	fungal res., low acrylamide, black spot bruise tolerance	5
Grapevine	fungal res.	1
Alfalfa	reduced lignine	1
Melon	fungal res.	1
Poplar	modify growth, stature and wood properties	1

Table 6.21 Notifications identified in the field trials database of the JRC about the use of cisgenesis and intragenesis.

TECH.	INSTITUTE/ COMPANY	MS	NOTIFICATION NUMBER	YEARS	PLANT	TRAIT	GENE INSERTED
CISG.	Wageningen University	NL	B/NL/07/01 B/NL/09/02	2007-2012 2010-2020	potato	late blight resistance	*Rpi-blb1*
CISG.	Stichting Dienst Landbouwkundig Onderzoek (DLO)	NL	B/NL/10/06	2011-2021	potato	late blight resistance	*Rpi-genes*
			B/NL/10/05	2011-2021	apple	scab resistance	*HcrVf2*
CISG.	University of Ghent	BE	B/BE/10/V1	2011-2012	potato	late blight resistance	*Rpi-vnt1*
CISG.	Teagasc, Oak Park	IE	B/IE/12/01	2012-2016	potato	late blight resistance	*Rpi-vnt1.1*
CISG.	Aarhus University	DK	B/DK/12/01	2012-2012	barley	improved phytase activity	*HvPAPhy_a*
INTRA.	AVEBE	NL	B/NL/07/05 B/NL/04/04 B/NL/03/04 B/NL/07/04	2008-2011 2004-2013 2004-2013 2008-2015	potato	reduced amylose content	*GBSS*

Grafting non-GM varieties onto GM rootstocks

Only the case of a non-GM scion grafted on a GM rootstock was investigated in the literature search. Identified publications on this topic were divided into two categories: i) publications describing the use of grafting for research purposes only (i.e. research on transfer of molecules between rootstock and scion) and ii) publications describing the use of the technique for plant breeding. Within the second category, 58 research papers have been identified about the transformation of rootstock plants for plant breeding purposes. However, the use of non-GM scion into the GM rootstocks is not specified in all publications. Since the focus of this study is related to the regulatory challenges posed by NPBTs, the papers were selected in which the use of GM rootstock for grafting of a non-GM scion was clearly specified. Twenty one research papers and three review papers belonging to this description were identified.

As illustrated in Figure 6.9, first publications about grafting on GM rootstock appeared in 1991. Most publications have been produced by authors from the EU (Table 6.22) and mostly from authors of academia (20 papers), while only 4 papers have been produced by joint institutions and no one only from industry. According to Table 6.23 institutions publishing the most relevant papers are from France (INRA and Université Angers), the US (Washington State University and other universities listed in Table 6.23) and the Netherlands (University of Wageningen and Centre for Plant Breeding & Reproduction Research), all academic.

According to scientific publications, mainly traits for disease resistance have been introduced in GM rootstocks (Table 6.24), in particular virus resistance in potato, grapevine, pea, cucumber and watermelon, and fungal resistance in potato and plum. Some other studies are focused on the improvement of rooting ability through transformation with *rol* genes of *Agrobacterium rhizogenes* in apple, rose and walnut. According to the remaining publications, the technique is applied in potato also for bacterial resistance, in tomato and orange to increase defence activity, in watermelon to improve robustness and in poplar to induce early flowering.

For grafting on GM rootstocks notifications for four different crops were identified in the field trials database (Table 6.25): for apples and pears with GM rootstocks with “improved rooting ability”, for grape vines with GM rootstocks resistant to the grapevine fanleaf virus, for orange trees with rootstocks resistant to *Phytophtora* and for citranges with rootstocks over-expressing an oxidase gene with the aim of modifying plant architecture.

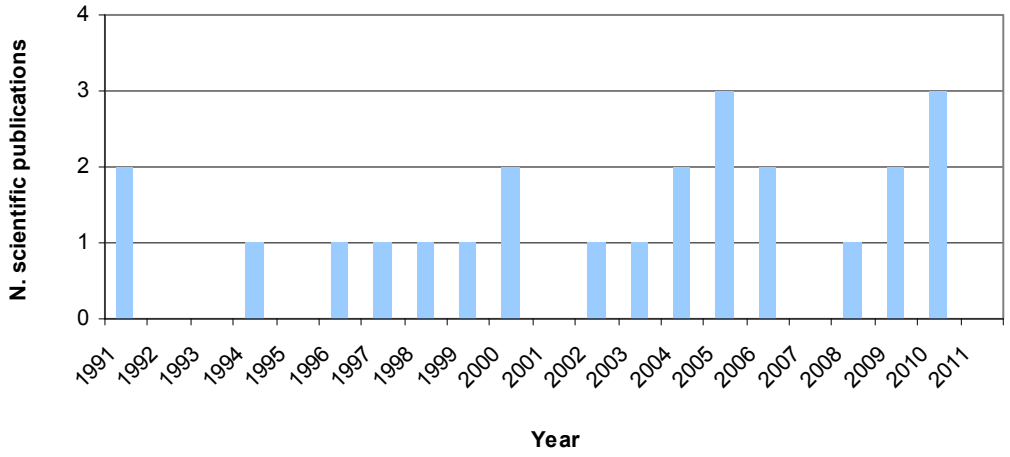

Figure 6.9 Development over time of scientific publications on grafting on GM rootstock

Table 6.22 Geographical distribution of scientific publications on grafting on GM rootstock. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications
EU-27	**13**
Netherlands	3
UK	3
Sweden	3
France	2
Germany	1
Finland	1
North America	**8**
USA	7
Canada	1
Asia	**6**
Korea	4
Japan	1
China	1
Other countries	**3**
Australia	1
New Zealand	1
Israel	1

Table 6.23 First four papers on RdDM wih the highest 5-year impact factor and institutions of authorship.

Research paper	Journal	5-year impact factor	Institution
Lambert and Tepfer (1991)	Nature Biotechnology	28.161	INRA (FR) Université Angers (FR)
McGurl *et al.* (1994)	PNAS	10.472	Washinghton State University (US)
van der Salm *et al.* (1998)	Journal of Experimental Botany	5.480	Centre for Plant Breeding & Reproduction Research (NL) University of Wageningen (NL)
Zhang *et al.* (2010b)	Journal of Experimental Botany	5.480	Northwest A&F University, College of Forestry (CN) Oregon State University (US) Mississippi State University (US) University of Minnesota (US)

Table 6.24 Plants and traits for which grafting on GM rootstock is used according to literature.

Plant	Traits	N. publications
Potato	Virus, bacterial and fungal res.	4
Apple	Rooting ability	3
Watermelon	Virus res., Robustness	3
Rose	Rooting ability	2
Grapevine	Virus res.	2
Tomato	Defence activity	1
Walnut	Rooting ability	1
Pea	Virus res.	1
Cucumber	Virus res.	1
Plum	Fungal res.	1
Poplar	Early flowering	1
Orange	Defence activity	1

Table 6.25 Notifications identified in the field trials database of the JRC about the use of grafting on GM rootstock.

INSTITUTE/ COMPANY	MS	NOTIFICATION NUMBER	YEARS	PLANT	TRAIT	GENE INSERTED
INRA	FR	B/FR/09/11/01 B/FR/04/05/01	2010-2014 2004-2008	grape grape (vinifera x berlandieri)	resistance to Grapevine fanleaf virus (GFLV)	coat protein (CP) gene of GFLV
IVIA	ES	B/ES/06/43 B/ES/08/03	2007-2017 2008-2018	carrizo citrange	modified plant architecture	GA20-oxidase
IVIA	ES	B/ES/08/04	2008-2018	sweet orange	tolerance to *Phytophtora citrophtora*	*PR P23*
Swedish University of Agr. Sciences	SE	B/SE/04/1227 B/SE/09/12183	2004 2010-2014	apple pear apple pear	improved rooting ability	*rolB*

6.2.4 Aggregated results

The whole literature search in the field of NPBTs as described in the previous paragraphs retrieved a total of 186 scientific publications. Figure 6.10 shows the distribution over time of the total number of publications identified. Most publications on new techniques were produced starting in the last decade, and the total number of publications seems to follow a growing trend, reflecting an increasing level of research activity in the field. The most recent plant breeding technique in terms of publication dates is TALEN technique.

According to the geographical distribution of all publications identified in NPBTs (Table 6.26 and Figure 6.11), the EU leads with 42.6% of the publications. Within the EU, the highest number of publications on NPBTs was produced by the Netherlands (12.6% of all publications). The EU is followed by North America in number of publications (32.7% of the total), mainly produced by the US (31.8% of the total). The majority of publications (77%) were produced by academic institutions, followed by industry (12.8% of publications) and by joint collaborations between academy and industry (10.2%).

The leading institutions on R&D of NPBTs were identified by analysing authorship of the retrieved publications. 168 institutions in total are active in the literature about NPBTs. Considering the absolute number of publications (Table 6.27), Wageningen University from the Netherlands is in first position. However, counting the number of techniques investigated (Table 6.27), the University of California is in leading position, covering five of the eight techniques analysed. We can observe that most institutions of the top ten are from the US, despite the EU dominance in total number of publications. Only two private institutions appear in the top list: J.R. Simplot Company from the US, involved in R&D of intragenesis, and the multinational company Pioneer Hi Bred International, having published about ODM, MGN techniques and RdDM.

Analysing the first ten papers on NPBTs in terms of highest impact factor (Table 6.28), what emerges is that the most relevant papers have been written on targeted mutagenesis in plants, in particular on ZFN and ODM techniques, which are the most advanced of the four. All publications on targeted mutagenesis in the top ten have been produced by US-based authors, both from academy and industry. Only RdDM and Grafting on GM rootstock appear in the top ten, and mostly with publications produced in EU-based academic institutes.

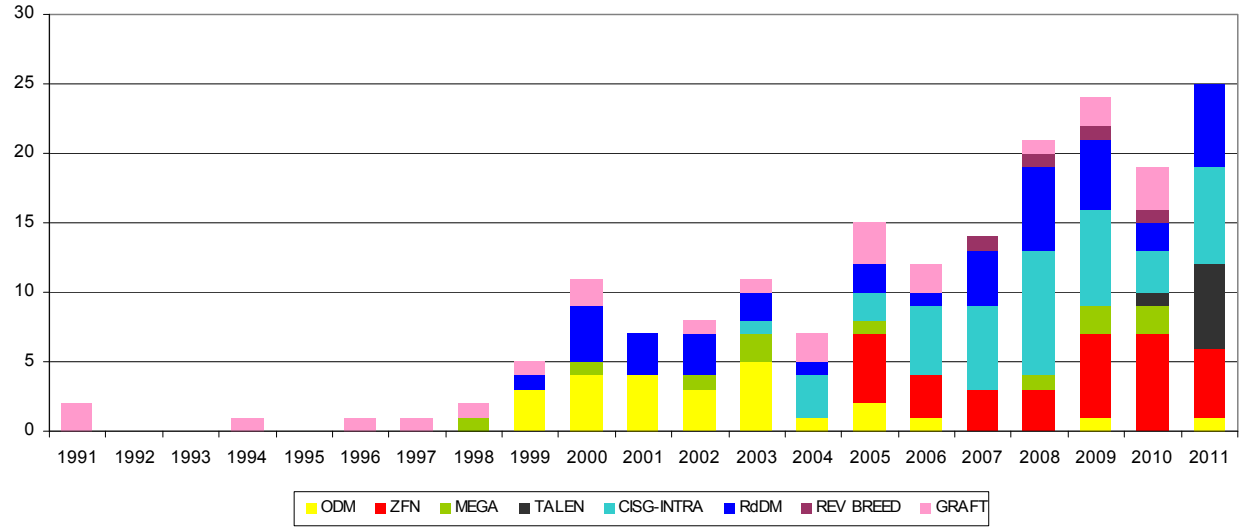

Figure 6.10 Development over time of scientific publications on NPBTs.

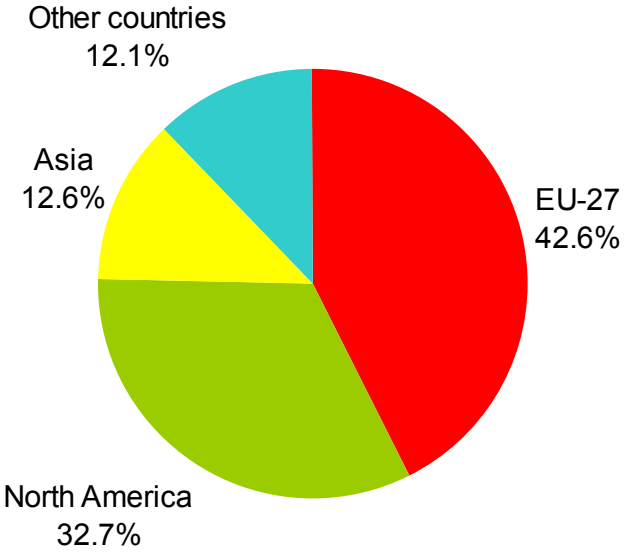

Figure 6.11 Geographical distribution of scientific publications on NPBTs.

Table 6.26 Geographical distribution of scientific publications on NPBTs. Each number refers to the number of papers with at least one author from the specific country.

Authors country	N. publications									
	ODM	ZFN	MGN	TALEN	CISG-INTRA	RdDM	REV BREED	GRAFT	TOTAL	% IN TOTAL
EU-27	**9**	**4**	**7**	**3**	**26**	**29**	**4**	**13**	**95**	**42,6**
Netherlands		1			17	4	3	3	28	12,6
Germany	6	2	5	3	2	3		1	22	9,9
Austria						12	1		13	5,8
UK	1				3	3		3	10	4,5
France		1	1			3		2	7	3,1
Italy					4	1			5	2,2
Belgium	2		1			1			4	1,8
Sweden								3	3	1,3
Cz. Republic						2			2	0,9
Finland								1	1	0,4
North America	**13**	**24**	**5**	**2**	**15**	**4**	**2**	**8**	**73**	**32,7**
USA	12	24	5	2	15	4	2	7	71	31,8
Canada	1							1	2	0,9
Asia	**2**	**4**	**0**	**3**	**3**	**9**	**1**	**6**	**28**	**12,6**
Japan	2	2				8		1	13	5,8
Korea		1						4	5	2,2
China				1		1	1	1	4	1,8
India		1			2				3	1,3
Saudi Arabia				2					2	0,9
Bangladesh					1				1	0,4
Other countries	**3**	**5**	**0**	**0**	**11**	**5**	**0**	**3**	**27**	**12,1**
Switzerland	1	2			4				7	3,1
Israel		3			1			1	5	2,2
Australia	1				1	2		1	5	2,2
New Zealand					2			1	3	1,3
Norway					2				2	0,9
Russia						2			2	0,9
Chile					1				1	0,4
Brazil						1			1	0,4
Un. Nations	1								1	0,4

Table 6.27 Most active institutions in NPBTs in plants, based on number of publications produced (second column from the right) and number of techniques covered (first column on the right). Each technique is represented by a letter. O:ODM, Z:ZFN, M:MGN, T:TALEN, C: Cisgenesis/Intragenesis, R:RdDM, B:Reverse Breeding, G:Grafting.

Institution	Sector	Country	City	N. publication	Techniques
University of Wageningen	Academy	NL	Wageningen	21	C,R,B,G
Austrian Academy of Sciences	Academy	AT	Salzburg	12	R
Iowa State University	Academy	US	Ames, IA	11	Z,T
J.R. Simplot Company	**Industry**	US	Boise, ID	10	C
University of California	Academy	US	Davis, CA	8	O,C,R,B,G
University of Minnesota	Academy	US	Minneapolis, MN	7	Z,T,G
Institute of Plant Genetics and Crop Plant Research (IPK)	Academy	DE	Gatersleben	7	O,M,R
Pioneer Hi-Bred International	**Industry**	US	Johnston	5	O,M,R
University of Delaware	Academy	US	Newark	5	O,R
Harvard Medical School	Academy	US	Boston, MA	6	Z

Table 6.28 First ten papers on NPBTs in terms of 5-year impact factor and institutions of authorship.

NPBT	Research paper	Journal	5-year impact factor	Institution
ZFN	Shukla *et al.* (2009)	Nature	36.235	Dow AgroSciences (US) Sangamo BioSciences (US)
ZFN	Townsend *et al.* (2009)	Nature	36.235	Phytodyne, Inc. (US) Iowa State University (US)
RdDM	Kanno *et al.* (2008)	Nature Genetics	33.096	Austrian Academy of Sciences (AT) University of Veterinary Medicine (AT) Boku University Vienna (AT)
ODM	Zhu *et al.* (2000)	Nature Biotechnology	28.161	Pioneer Hi-Bred International, Inc (US)
GRAFTING	Lambert and Tepfer (1991)	Nature Biotechnology	28.161	INRA (FR) Université Angers (FR)
ZFN	Sander *et al.* (2011)	Nature Methods	20.454	Massachusetts General Hospital (US) Harvard Medical School (US) University of Minnesota (US) Yale University School of Medicine (US) Iowa State University (US) Broad Institute (US)
ZFN	Maeder *et al.* (2008)	Molecular Cell	14.202	Massachusetts General Hospital (US) Charite´ Medical School (DE) Iowa State University (US) University of Iowa (US) Harvard Medical School (US) UT Southwestern Medical Center (US)
RdDM	Sijen *et al.* (2001)	Current Biology	10.881	BioCentrum Amsterdam, Vrije Universiteit (NL)
RdDM	Melnyk *et al.* (2011)	Current Biology	10.881	University of Cambridge (UK)
ODM	Beetham *et al.* (1999)	PNAS	10.472	Boyce Thompson Institute for Plant Research, Cornell University (US)

6.3 Safety and Regulatory issues on new techniques identified in literature

The need for regulating products of NPBTs may stem from safety issues (food, feed or environmental safety). This section is dedicated to the aspects of regulatory issues and safety issue related to NPBTs that already emerged in literature. The papers identified on NPBTs reported in section 6.2 and additional literature identified throughout the analysis of the technique have been analysed for their content related to safety and regulatory aspects.

Table 6.29 provides references to publications and reports identified as relevant for the food, feed and environmental safety of each NPBT. The table also include information on the main conclusions or issues discussed for each publication. As food, feed and environmental safety aspects of NPBTs are closely related to the regulatory issues and both topics are frequently discussed in the same publications, all related information are included in Table 6.29.

Reports on discussions about food, feed and the environmental safety of the NPBTs are available from the Netherlands and Belgium. One report (in English) from the Dutch Commission on Genetic Modification (COGEM) from 2006 (COGEM, 2006b) covers all NPBTs with the exception of ZFN technique and cisgensis and intragenesis. The Belgian Biosafety Advisory Council (BAC) has evaluated the use of "Targeted Gene Repair" which covers ODM (BAC, 2007).

One scientific paper from Wageningen University (WUR) in the Netherlands evaluates food, feed and environmental risk of crops derived through all NPBTs except ZFN technique and RdDM (Schaart J.G. and Visser, 2009). In addition review papers were identified in which scientists discuss safety aspects of NPBTs. Safety aspects are also frequently discussed in the context of research related to technical aspects of the NPBTs. A small number of analysed papers relate to research on specific safety aspects of NPBTs, e.g. the gene flow from GM rootstocks to the soil.

In the course of this study on NPBTs, in addition to the information of scientific literature about the regulatory and safety issues of the techniques, also experts' opinions have been collected. The next sections attempt to summarise the opinions collected about the technical reasons for which each technique can be considered as included in or excluded from Directive 2001/18 on GMOs. The scope of the section is to understand the grounds of the regulatory uncertainty that currently surrounds NPBTs.

6.3.1 Targeted mutagenesis: ODM, ZFN, MGN, TALEN

Reasons for excluding the techniques from the scope of Directive 2001/18

Classical mutagenesis obtained through the use of chemicals or irradiation (as described in section 1.2.4) is included in the list of Annex IB of Directive 2001/18. This means that mutagenesis is recognised as a technique of genetic modification but the products are excluded from the scope of the GMO regulation. For similarity of results with classical mutagenesis, the products of targeted mutagenesis could also be listed in the same annex, and would present the additional advantage of site-specific targeting.

Reasons for including the techniques in the scope of Directive 2001/18

Techniques of recombinant DNA are included in Annex 1A part 1 of Directive 2001/18 and thus their products are considered as GMOs included in the scope of the Directive. In the cases of ZFN, MGN and TALEN techniques, recombinant DNA is employed: the sequences to be expressed are assembled *in vitro* before to be transferred to the plant cells. In the case of ODM, synthetic oligonucleotides are delivered to the plant cells to directly operate at the plants DNA. Therefore, the question arises if to consider those oligonucleotides as recombinant DNA or not.

Regarding ZFN, MGN and TALEN technique, the genes delivered for the expression of nucleases could either be expressed transiently or be integrated. In the latter case, there would be an intermediate step of presence of the transgene, which should be finally segregated out. For this case, the reasons of exclusion or inclusion are analysed in section 2.4.6 on negative segregants.

For the case of site-specific gene integration (ZFN 3, MGN 3 and TALEN 3), an entire gene is integrated, which can proceed from a cross-compatible species or from a different organism. In the first case, the reasons for exclusion or inclusion would be similar of the ones for cisgenesis or intragenesis. In the second case, the only reason for not considering the resulting plants as GMOs, would be perhaps in case that site-specificity is demonstrated to represent a big advantage in terms of safety.

In addition to the previous observations, literature on targeted mutagenesis through the use of modified nucleases (ZFN, MGN and TALEN) often described the concerns about the rate of off-targets breaks, which is related to the level of specificity (usually the length of the recognised sequence) of the nuclease. These unwanted breaks can convert in additional mutations, with unpredictable effects. Therefore, this issue should be taken into account in the evaluation of the safety of the final product. However, this issue could also be considered as comparable with the

characteristic of classical mutagenesis techniques to create random mutations in the whole plant genome, with no possibility of predict number and sites of the mutations beforehand.

6.3.2 Negative segregants: RdDM, Reverse breeding and Early flowering

The distinction between process- and product- based GMO definition plays a fundamental role in determining if these techniques fall or not under the scope of GMO regulation, since the final products are clearly free of any transgenic sequence but transgenesis is employed in the breeding process. Additionally, another question should be raised in order to decide about the classification of the obtained plants: if the offspring of GM plants is considered as GMOs even in the absence of the inserted genetic construct.

Reasons for excluding the techniques from the scope of Directive 2001/18

If non transgenic plants segregation is performed correctly, the final products of reverse breeding and early flowering techniques are indistinguishable from products of conventional breeding. In the case of reverse breeding, the obtained plant should perfectly reproduce the initial hybrid and in the case of early flowering, the transgene only intervenes to accelerate the breeding process and not to modify any other gene sequence.

In the case of RdDM a gene expression is silenced but only at epigenetic level, that in principle could be provoked also by environmental factors.

Reasons for including the techniques in the scope of Directive 2001/18

The intermediate transgenic plants are obtained through transformation techniques in the same way as plants currently regulated under the scope of Directive 2001/18.

6.3.3 Variants of plant transformation techniques: Cisgenesis, Intragenesis and Grafting non-GM varieties onto GM rootstock

Intro: since the two techniques included in this group have been analysed in separate publications and since the motivations behind the reasons to include or exclude these techniques form GMO regulation are based on different concepts, the two techniques are considered in separate section, as follows.

Cisgenesis and Intragenesis

Reasons for excluding the techniques from the scope of Directive 2001/18

In both cisgenesis and intragenesis, the DNA sequences inserted in the host plant originate from cross-compatible plant species only. It can be said that there is a probability of obtaining the same result also through conventional crossing with the same compatible species. In cisgenesis an entire piece of DNA is extracted from the compatible plant and transferred to the host, while in intragenesis, several DNA sequences can be combined, also originating from different compatible species and also in antisense direction. Therefore, the probability of obtaining the same resulting product by conventional breeding is clearly much smaller through intragenesis than cisgenesis. Additionally, cisgenesis and intragenesis do not introduce unwanted sequences as in the case of conventional crossing, in which inevitably more DNA is introduced into the target variety, than the specific wanted gene. This phenomenon is called linkage drag.

The defenders of the de-regulation of cisgenesis and intragenesis from the GMO legal framework, state that the absence of foreign sequences in the final product, guarantees a higher safety compared to transgenic plants, since they lack new unknown DNA that might lead to the production of allergens, toxins or anti-nutritional compounds and do not contain selectable markers. There are several data about public opinion more favourable towards the products of cisgenesis than transgenesis, feeling that they are more acceptable from a safe and ethical point of view.

Reasons for including the techniques in the scope of Directive 2001/18

Both cisgenic and intragenic plants are obtained through transformation techniques as transgenic plants (mainly *Agrobacterium* mediated or particle bombardment). This implies that recombinant DNA is employed (as for techniques of Annex IA part 1) and that the delivered DNA sequence integrates in a random (non site-specific) place of the plant genome. The genomic area in which the gene integrates normally influences the level of expression of the gene. This phenomenon is called “position effect” and causes variation in the possible expression level of the new gene. Additionally, the random integration of a new sequence might influence the pre-existing gene sequences and influence their expression, e.g. by interrupting an ORF (open reading frame) and thereby silencing the corresponding gene. Also, new ORFs can be created by the insertion of a new sequence.

As illustrated in the definition of cisgenesis (section 1.4.2), there is a general disagreement among experts regarding the inclusion of T-DNA borders in the definition of cisgenic plants.

Finally the NTWG decided to separate two different categories: cisgenesis with and without T-DNA borders.

According to the EFSA Scientific opinion on the safety of cisgenic and intragenic plants (EFSA, 2012), similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants, because of new combination of genetic elements. EFSA states that no new guidelines for the risk assessment of cisgenic and intragenic plants are needed, since the guidance documents used for transgenic plants (EFSA, 2010, 2011) are applicable and do not need to be further developed. EFSA GMO Panel states that, on a case-by-case basis, lesser amounts of event-specific data could be required for the risk assessment of cisgenic or intragenic products. Regarding T-DNA borders, the GMO Panel concludes that similar sequences can be found in different plant species. Therefore, any hazards related to these sequences would not differ from those in conventional plant breeding.

Grafting non-GM varieties onto GM rootstocks

Reasons for excluding the techniques from the scope of Directive 2001/18

The fruits of the non GM scion can be considered as non transgenic and therefore do not need to be subject of food/feed safety assessment. A part from not carrying any foreign sequence fruits of non-GM scion would not produce any GM pollen, therefore avoiding the legal issues of co-existence.

Reasons for including the techniques in the scope of Directive 2001/18

For the obtainment of the GM rootstock, transformation techniques are employed, thus the final result is transgenic by definition. Even if a non GM scion is grafted on the GM rootstock, the final plant, if considered as a whole, is transgenic. In any case, the cultivation of the plants obtained through grafting on GM rootstock implies their release into the environment, i.e. the transgenic part will get into contact with the environment. Therefore, according to the rules established for GMOs, the environmental assessment of the rootstock should be performed.

Regarding the scion and, in particular, the fruits, no foreign DNA sequence are present in the genome. However, it should be demonstrated if molecules like proteins or RNAs that originate from the transgene, can be transferred into the scion and if they can influence on the characteristics of the fruits. Several studies have been produced on the molecular trafficking

between rootstock and scion, but the results are not homogeneous (Sonoda and Nishiguchi, 2000; Dutt *et al.*, 2007; Stegemann and Bock, 2009; Youk *et al.*, 2009).

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

NPBTs	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
Heselmans (2011)			X	Heselmans (2011) illustrates the current legal uncertainty about NPBTs in the EU. The author states that the uncertainty is blocking the business programmes of EU companies, especially small companies, and that the classification of the techniques as GM techniques would represent an advantage of multinationals over small companies. On the other side, NGOs like Greenpeace are pushing to have cisgenic plants classified as GMOs.
Kuzma and Kokotovich (2011)			X	Kuzma and Kokotovich (2011) describe the regulatory uncertainty around the group of targeted mutagenesis techniques in plants. They illustrate the differences, from a regulatory point of view, among the use of these techniques for creating site-specific mutations, for targeted insertion of transgenes or cisgenes and for deletion of entire sequences and discuss regulatory implications.
Lusser and Rodriguez-Cerezo (2012)			X	The report of Lusser and Rodriguez-Cerezo (2012) provides a short overview of the regulatory framework for biotechnology derived crops in six countries, mainly focusing on the legislation and GMO definitions. Additionally, the approaches for NPBTs in the six countries are provided together with a summary of considerations and decisions for specific groups of NPBTs.
Giddings et al. (2012)			X	According to Giddings et al. (2012), EU legislation on plant biotechnology should not be so strict with NPBTs, since the consequence would be a pernicious effect on applied plant science in Europe.
Waltz (2012)			X	Waltz (2012) reports that plant breeding companies, especially small companies, focus on the development of NPBTs that could circumvent the very strict requirements for GMOs. Targeted mutagenesis, negative segregants, chimeric grafting and all native transformation (when performed through biolistic) seem to have the chance of avoiding the whole process of authorisation in the US, which is foreseen for GM plants carrying "plant pest" sequences.
Mba et al. (2012)			X	According to Mba et al (2012), policy regulations that are expensive to comply with and public perceptions, rather than the ability to innovate, are holding back the unleashing of the incredible advances of science and technology in crop improvement.
Podevin et al. (2012)			X	Podevin et al. (2012) illustrate the situation of regulatory uncertainty around NPBTs and describe the regulatory challenges that need to be addressed urgently to establish an appropriate regulatory framework that is functional over time, stimulates innovation while building consumer trust and avoid disparities between equal products. The authors strongly highlight the need for international harmonisation in the regulatory system in plant breeding.

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

ODM	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The COGEM 2006 report concludes that there is a very small chance that unintended sequence modifications could be caused by ODM besides the intended sequence modifications. This should be taken into account for the risk assessment.
Schaart and Visser (2009)	X	X		The COGEM report 2009 (commissioned to Schaart and Visser) recommends treating organisms obtained through ODM the same way as organisms produced by chemical or physical mutagenesis.
BAC (2007)	X	X	X	The authors of both reports consider that there are scientific arguments for having ODM excluded from the scope of the EU Directives on GMOs. The recommendation is based on a discussion of the legal situation and the comparison of crops obtained by ODM with crops generated through irradiation or chemical treatment. The high specificity of the technique, but also the need of further studies to improve the efficiency and effectiveness are taken into account.
Breyer et al. (2009)	X	X	X	
Oh et al. (2001)			X	The paper reports preliminary discussion about the classification of ODM products the same way of those obtained through classical mutagenesis and not through transgenesis.

ZFN	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
Wright et al. (2005)			X	The paper reports preliminary discussion about greater acceptance of targeted mutagenesis compared to plant transformation and about the difficulties foreseen for classifying this technique.
de Pater et al. (2009)	X	X		Several papers about the use of ZFN technique for targeted mutagenesis highlight that a certain risk of cytotoxicity is associated to the technique, due to the activity of ZFNs at off-target sites. This drawback has to be corrected by increasing the specificity of the ZFN.
Urnov et al. (2010)	X	X		
Zhang et al. (2010a)	X	X		
Gupta et al. (2011)	X	X		
Ramalingam et al. (2011)	X	X		

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

Tzfira et al. (2012)	X	X		
DeFrancesco (2011)	X	X		According to De Francesco (2011), ZFN technique implies the risk of off-target cleavage and resulting toxicity when zinc-finger domains are fused with Fok1 nuclease. Off-target cleavage is caused in part by nonspecific DNA binding of the zinc-finger domains and in part by the Fok1 nuclease.
Gruskin (2012)			X	Gruskin (2012) states that the very complex process of deregulation that is in place in the USA for biotech crops could be avoided for products of ZFN and TALEN techniques, due to their similarity with products of conventional mutagenesis. USDA-APHIS is currently considering the regulatory status of both techniques.

MGN	**Issues covered**			**Main conclusions, discussions or remarks**
	Food and Feed Safety	**Safety for the environment**	**Regulatory issues**	
D'Halluin et al. (2008)	X	X		According to D´Halluin et al (2008), site-specific integration through MGN techniques is a way to improve the quality of transgenic plants and to avoid the risks of direct DNA transfer methods: mutations arising from random integration and gene silencing by multiple copy integration. Therefore, it would improve the safety of the final product.

TALEN	**Issues covered**			**Main conclusions, discussions or remarks**
	Food and Feed Safety	**Safety for the environment**	**Regulatory issues**	
Li et al. (2011)	X	X		According to Li et al (2011), TALENs cause no detectable cytotoxicity and minimal levels of undesired genetic mutations in the treated organisms.
Mahfouz and Li (2011)	X		X	According to Mahfouz and Li (2011), the TALENs molecular scissors techniques have the potential to allay concerns about foreign DNA and random integration and facilitate the deregulation of GM crops.
DeFrancesco (2011)	X	X		DeFrancesco (2011) states that TALEN technique compared to ZFN technique causes less cytotoxicity related to off-target events, even when fused with wild-type FokI nuclease.
Gruskin (Gruskin, 2012)			X	According to Gruskin (2012), the very complex process of deregulation that is in place in the USA for biotech crops could be avoided for products of ZFN and TALEN techniques, due to their similarity with products of conventional mutagenesis. USDA-APHIS is currently considering the regulatory status of both techniques.

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

RdDM	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The COGEM 2006 report observes that it is too early at this time to make judgements on any environmental risks of epigenetic mutants as too little is known about the stability of epigenetic changes and the mechanisms of inheritance. They also state that it is currently not clear to what degree plants obtained wit RdDM are subject to GMO legislation.

REVERSE BREEDING	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The authors of the COGEM 2006 report are of the opinion that risk analysis required for transgenic plants, is not needed for plants obtained through reverse breeding. This conclusion is based on the fact that hybrid plants obtained through reverse breeding do not contain recombinant DNA and do not have any new genetic characteristics. Therefore no new open reading frames can be created through which toxic or allergenic products could be formed. The COGEM 2006 report also states that reverse breeding makes use of genetic modification and that therefore, according to the European legislation, a crop produced through reverse breeding would be recognised as a GMO. The COGEM 2006 report, however, recommends that plants that are acquired using the technique of reverse breeding should be handled as non-GMOs because they do not have any new characteristics compared to the starting heterozygous plant,
Schaart & Visser (2009)	X	X		Schaart & Visser's report (2009) concludes that food and feed of crops obtained through reverse breeding are as safe as products from the original heterozygous lines, because F1-hybrids obtained by crossing of reverse breeding-derived parental lines do not contain any genetic modification-related DNA sequences and a possible RdDM that is transmitted to the offspring will only have an effect on meiotic recombination. For the same reasons the consequences for the environment will be in principle similar to those of parental lines and F1-hybrids obtained by conventional breeding.

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

CISGENESIS, INTRAGENESIS	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
COGEM (2006a)	X	X		In 2006 COGEM published a report on the ethical and societal aspects of cisgenesis.
Myska (2006)		X	X	Myska (2006) discusses ethical aspects of intragenic versus transgenic modification in plants.
Schouten et al. (2006a)	X	X	X	Jacobsen, Schouten and co-workers discuss in several publications regulatory issues and safety aspects of cisgenesis. They regard cisgenic plants as comparable to conventionally bred plants and propose the exemption from the GMO legislation in a step by step approach. They base their conclusions on the following arguments: i) Cisgenic plants only contain genetic elements that belong to the gene pool of traditional breeding. ii) Cisgenesis is a way to avoid linkage drag. iii) Transformation without marker genes in the commercialised products is possible. iv) Random insertion and mutations at insertion site are common phenomenona also in traditional breeding.
Schouten et al. (2006b)	X	X	X	
Jacobsen and Schouten (2007)			X	
Jacobsen and Schouten (2008)	X	X	X	
Schouten and Jacobsen (2008)			X	
Jacobsen and Schouten (2009)	X		X	
Park et al. (2009)	X		X	
Schouten et al. (2009)	X	X	X	
Val Gidding (2006)			X	Val Gidding 2006 provides a critical reply to Schouten et al 2006b. He disagrees with the statement that cisgenic plants are safer than transgenic and therefore should be exempted from GMO regulation.
De Cock Buning et al. (2006)			X	De Cock Buning et al. 2006 challenge Schouten et al. 2006 a and b with regard to the terminology (cisgenesis) used and the conclusions.
Lammerts Van Bueren et al. (2007)			X	Lammerts Van Bueren et al. (2007) discuss cisgenesis and reverse breeding in the context of organic farming. They regard products obtained through these techniques as not acceptable in organic farming because of ethical reasons (respect of naturalness and integrity of all organisms).
Rommens et al. (2007)	X	X	X	Rommens et al. (2007) argue that intragenic plants are at least as safe as those developed through traditional methods, because they lack new unknown DNA that might lead to the production of allergens, toxins or antinutritional compounds and do not contain selectable markers.
Rommens (2007)	X	X	X	
Conner et al. (2007)			X	Preliminary discussion about the difficulties in classifying the products of intragenesis, due to their similarity with the products of conventional breeding and the difficulties in detecting them.

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

Kok et al. (2008)	X		X	In a publication on 'Comparative safety assessment of plant-derived foods', Kok et al (2008) discuss inter alia the safety aspects of crops obtained through cisgenesis. A distinction is made between introduced genes coming from a crop that is already used as a food source and genes coming from plant wild relatives without a "history of safe use". In the latter case, they recommend to asses the safety of the newly introduced sequences and protein(s).
Russell and Sparrow (2008)	X	X	X	Russell and Sparrow (2008) compare the regulatory system in US, Canada, Europe, Australia and New Zealand applicable for GM plants and the way it is applied or could be applied to intragenic plants. They also discuss environmental and food safety issues. The authors recommend classifying intragenic plants as GMOs.
Haverkort et al. (2008)			X	Haverkort et al. 2008 discuss the issue of regulating cisgenic crops in the context of a study on "Societal Costs of Late Blight in Potato and Prospects of Durable Resistance Through Cisgenic Modification".
Schaart and Visser (2009)	X	X		Concerning the consequences for food and feed safety and the environmental consequences of cisgenesis Schaart and Visser (2009) conclude that cisgenic plants can be regarded as similar to conventionally bred plants under the following conditions: It has to be proven that the variation of the cisgene expression is within the expression variation of the corresponding gene in its natural genomic context, that no genes of the recipient have been mutated as a result of the integration, and that T-DNA borders have not become part of an open reading frame. Conventionally bred plants can be used as baseline for the risk assessment. In the case of intragenesis, the COGEM 2009 report concluded that no general statement about the consequences for the environmental and for food and feed safety can be made. Because of the position effect and the recombinant nature of the used gene, variability in expression of the gene has to be expected. Therefore the evaluation of the risk should be carried out on a case-by-case basis. Conventionally bred plants can be used as baseline. In the case intragenesis is aimed at silencing native genes, the intragenic plants may be comparable to plants with knock-out mutations. Such plants can be used as baseline.
Jacobsen and Schaart (2009)	X			Jacobsen & Schaart (2010) evaluate the biosafety of T-DNA border sequences from *Agrobacterium* when present in cisgenic crops. They conclude that the risks for food and feed of the T-DNA borders are negligible compared to the risks of conventional breeding and mutation breeding which are regarded as suitable baseline for the risk assessment of transgenic plants.
Akhond and Machray (2009)	X			Akhond and Machray (2009) report the different opinions of scientists about cisgenic and intragenic products. From one side they describe the risk related to T-DNA sequences and random gene integration and from the other side the similarity of cisgenesis and intragenesis with conventional breeding.
Prins & Kok (2010)	X		X	Prins & Kok (2010) from the Institute of Food Safety of Wageningen University (RIKILT) report on a project on the food and feed safety of cisgenesis carried out on request of the Dutch Ministry of Housing, Spatial Planning, and the Environment (VROM). They conclude that the existing knowledge on newly expressed proteins in cisgenic/intragenic plant varieties may lead to reduced requirements in specific aspects of the food and feed safety assessment (already under the current

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

				legislation). They are of the opinion that there is no scientific basis for a general reduction of requirements for the risk assessment for cisgenic plant varieties. They base their conclusion on arguments such as the following: i) A watertight definition of cisgenesis/intragenesis is not possible. ii) Not all genes from the species' own gene pool necessarily have a 'history of safe use'. iii) The insertion of genes can cause unintended effects (insertional mutagenesis).
Polanco et al. (2010)				Polanco et al (2012) state that cisgenic plants are safer for the environment and for usage in food or feed, compared to transgenic plants. Additionally cisgenic plants are described as important for sustainability and well accepted by consumers.
Eurobarometer (2010) Gaskell et al. (2011)	X			According to Eurobarometer 2010, EU consumers are much more favourable about cisgenic than transgenic food, due the perception of a healthier food with no pesticide and no side effects of foreign genes.
Bruening (2011)	X	X	X	Bruening (2011) states that the US regulatory system for the authorisation of GMOs is not well balanced between GM techniques and conventional breeding. According to him, potentially unsafer plant breeding techniques that are considered as conventional are not subject of the same strict assessment. He also attempts to experimentally demonstrate that cisgenic products are not safer than transgenic products. According to the author the insertion of genes taxonomically closer increases the probability of unwanted interaction with endogenous proteins, and consequently the rate of possible unintended effects of transformation is higher.
EFSA (2012)	X	X	X	The EFSA GMO Panel considers that the EFSA Guidance documents available for the risk assessment of GMOs are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants and for performing an environmental risk assessment and do not need to be developed further. It can be envisaged that on a case-by-case basis lesser amounts of event specific data are needed for the risk assessment. The EFSA GMO Panel compared the hazards associated with plants produced by cisgenesis and intragenesis with those obtained either by conventional plant breeding techniques or by transgenesis. The Panel concludes that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants.
Holme et al. (2012)	X	X	X	Holme et al (2012) describes cisgenesis and intragenesis techiques and illustrate the issues about regulatory uncertainty and consumer perception of these techniques compared with trasgenesis.
AGES (2012)	X	X	X	The Austrian Agency for Health and Food Safety (AGES) presents an overview on the application of cisgenesis and intragenesis in plant breeding and the evaluation of potential consequences in different legal scenarios concerning detection, traceability, labelling, and risk assessment.

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

GRAFTING	Issues covered			Main conclusions, discussions or remarks
	Food and Feed Safety	Safety for the environment	Regulatory issues	
Vigne et al. (2004)		X		Vigne et al. (2004) carried out a field safety assessment which showed that rootstocks expressing the coat protein (CP) of Grapevine fanleaf virus (GFLV) do not favour the development of virus recombinants to detectable levels.
COGEM (2006b)	X	X	X	The COGEM 2006 report points out that it has to be considered if a graft is in a legal sense two different plants or one plant. They conclude that the GM rootstock clearly has to be subject of an environmental risk assessment, since it will be grown in the field. However, they state that it is unclear whether upper stems and the products of upper stems that have been grafted on GM rootstocks should be seen as GM. In addition, although they do not expect the presence of a transgene in the upper plant parts, there is the possibility that upper parts might display changed characteristics or that molecules produced in the transgenic rootstock such as proteins, RNA or other metabolites are transported to the upper parts. This has to be taken into account when carrying out a risk assessment for these crops which should be done on a case-by-case basis.
Kim et al. (2008)		X		Kim et al. (2008) carried out an evaluation of gene flow from GM plants for rootstock to wild type plants for determination of isolation distances
Schaart and Visser (2009)	X	X		The COGEM 2009 report notes that little is known about the transport of molecules (e.g. RNAi, proteins and metabolites) from a GM rootstock to the scion and the possible consequences in the scion. According to authors, further research is necessary on this subject before general conclusions can be drawn. They therefore recommend a case-by-case consideration of the food and feed safety of products from scions grafted on GM rootstocks. Concerning the consequences for the environment, the following issues should be taken into account additionally: In case RNAi-mediated silencing of the rootstock has led to RdDM of the target genes in the non-GM scion, the methylation-related phenotype can occasionally be stably inherited by the next sexual generation. The absence of silencing effects in the offspring should be proven before releasing the crop into the environment. Also the possible interaction of the GM rootstock with soil microoganisms has to be taken into account.
Hemmer et al. (2009)		X		Hemmer et al. (2009) studied the environmental impact of transgenic rootstocks expressing the coat protein (CP) of Grapevine fanleaf virus (GFLV). They showed that rootstocks expressing CP do not promote the emergence of GFLV variants, after 3 years.
Yi et al. (2009)		X		According to Yi et al (2009), crop plants interact with soil communities to form strong links and influencing agro-ecosystems, therefore soil microbial diversity must be adequately assessed to determine any environmental risk of GM rootstocks.

Table 6.29 Relevant literature on safety and regulatory issues related to NPBTs.

Youk et al. (2009)	X			Youk et al (2009) analyse the transgene integration in the GM rootstock and verified the absence of transgene sequences in the scion or other molecules moving from the rootstock to the scion. According to the authors, their results will constitute an important consideration in the determination of the food safety of watermelon generated from grafting of transgenic rootstock.
Smolka et al. (2010)	X			According to Smolka et al (2010), the use of GM rootstocks in combination with non-transgenic scion cultivars may circumvent the food safety issue if transgenes or their products are not present in scion fruits.
Haroldsen et al. (2012)		X	X	Haroldsen et al (2012) describe the uncertainties about the regulatory status of products of grafting on GM rootstock. A part from the transgene-free fruits, another regulatory advantage of these products would be the avoidance of GM pollen flow. There are still uncertainties however about molecules traffic between rootstock and scion, which is considered an important issue for the regulatory decision. The authors also illustrate the differences between the US and the EU system and the issues that might be raised by the import into the EU of unlabelled fruits from non GM scion.

6.4 Conclusions

In conclusion, what emerged from the literature search is that the field of NPBTs is rather young, publications starting only ten years ago, and the number of publications is growing quickly.

Considering individual techniques, the highest number of publications was identified for cisgenesis and intragenesis (43 papers), followed by RdDM (40 papers), ZFN techniques (32 papers) ODM (25 papers) grafting on GM rootstock (24 papers), and MGN techniques (11 papers). Only seven papers were identified for TALEN techniques and four papers for reverse breeding. Both are also the most recent techniques according to publication dates. Considering groups of techniques, targeted mutagenesis is clearly the group with the highest number of publications (75 papers in total) and this reflects a clear interest in these techniques for precise plant genetic modification.

Public research institutions from European countries have produced the highest number of publications followed by the US. The EU leads in publications on cisgenesis/intragenesis, reverse breeding, MGN and TALEN techniques, RdDM and grafting on GM rootstock. The US has the highest number of papers on ZFN technology and ODM. However, by analysing the leader institutions in terms of number of publications and the authorship of the papers with the highest impact factor, US-based authors have clearly a dominant role. This is due in particular to the relevance of the publications on ODM and ZFN techniques, which possess the highest impact factor. This data is representative of the relevance of research activity in the US and will be compared in the next section with the data of industrial application derived from patent search. The ten leading institutions publishing research on NPBTs are mostly public institutes with the exception of two.

The proof of concept of the NPBTs has been achieved mostly by introducing herbicide tolerance and disease resistance traits. There are substantial differences among techniques in terms of their current applicability to crop species. Among mutagenesis technique, ODM has been proven to work on a variety of crop plants (that is, maize, rice, wheat, oilseed rape and even banana) and ZFN techniques on model plants but also in maize, soybean and the ornamental plant petunia, whereas other more recent mutagenesis approaches, such as MGN and TALEN techniques, have been mainly reported in model plants. Grafting on GM rootstock, cisgenesis and intragenesis, on the other hand, have already been used on several crop plants because they rely on existing tools for genetic modification (transformation by *Agrobacterium* or particle bombardment). RdDM has been applied in a few crop plants (maize, potato, tomato, rice, carrot

and petunia) for the silencing of several marker genes. Finally, reverse breeding, has not yet been the subject of any scientific research papers, only a handful of reviews.

Although the majority of publications on NPBTs are recent, several documents have been already produced on safety and regulatory issues related to these techniques. This is mainly due to the concern of scientists and breeders about the regulatory constraints that might be established on these techniques and that could therefore hinder their development.

The highest number of publications on safety and regulatory issues has been produced for cisgenesis and intragenesis techniques, in particular by authors from Wageningen University (developers of cisgenesis) and Simplot Company (developers of intragenesis). Many publications have also been produced on safety and regulatory aspects of targeted mutagenesis, in particular for ODM and ZFN techniques. Fewer publications are available to date for the other NPBTs.

Several publications identified on safety and regulatory aspects of NPBTs report the wish of authors of having the new technique excluded from the scope of GMO regulation (in the EU or US). Only few publications report scientific opinions concerned about safety issues (such as Lammerts Van Bueren *et al.*, 2007; Russell and Sparrow, 2008; Prins and Kok, 2010 among others) and, on the other side, some authors express the need to regulate certain products of conventional breeding in a similar way as the products of biotechnology (Kok *et al.*, 2008; Bruening, 2011).

Chapter 7. Results: patent analysis of NPBTs

7.1 Introduction

Whereas the literature search is useful for assessing the current knowledge about new techniques, it does not provide insight into industry activities since most published data come from academic institutions. Therefore, a patent search on NPBTs was performed in addition to the literature analysis to provide an overview of the applications for inventions related to NPBTs. A patent landscape analysis based on the number of patents per technique, in addition to a citation analysis, can identify the main actors interested in the commercial exploitation of a technique and its potential applications.

The patent search on NPBTs was finalised in September 2012. Because patent applications are normally published 18 months after filing, only patents filed by the beginning of 2011 are included in the findings. The description of the methodology followed in the patent search is provided in section 5.3 and described in more details in Parisi et al. (2012) and the whole list of patents identified per each technique is provided in Annex II. Both patent applications and granted patents were analysed, therefore the word patent is used for both cases. Each patent listed represents all members of its patent family. A patent family is defined as a set of patents—taken in various countries—that protect the same invention (Parisi *et al.*, 2012).

The following sections will present an overview of the information extracted from patents identified per technique in terms of number and priority dates, country and sector (public or private) of affiliation of the assignees. An analysis of citation has been performed to identify the presence of key patents in each field and claims have been thoroughly checked to identify indications about specific products obtainable through the patented method. When possible, information retrieved from Institutions' official Websites have been reported to integrate the information about products close to commercialisation.

7.2 Patents in NPBTs

The information extracted from the patent search in the field of NPBTs is presented in the next sections first technique by technique and finally as aggregated data for the whole field. As for literature information, the data for “targeted mutagenesis” techniques has been aggregated and analysed separately due to the high homogeneity of this group of techniques, both from the point of view of scope and of technical procedure.

7.2.1 Targeted mutagenesis: ODM, ZFN, MGN, TALEN

As for scientific literature (see section 6.2.1), several patents on targeted mutagenesis techniques claim their application in the field of medical biotechnology, and in particular in human and animal cells for the correction of point mutations that cause severe genetic diseases or for the insertion of gene of medical interest, which could replace defective genes.

Different classes of patents on targeted mutagenesis resulted from the search: patents specifically directed to plants, patents specifically directed to other organisms, like human or animal cells, and patents with broader claims that comprise more groups of organisms, including plants. Since the objective of the search is to cover all patents that are related to the use of targeted mutagenesis techniques in plants, both the findings belonging to the first and the third categories were included in the final list of results. As in the scientific literature search, the patent search also included patents describing targeted gene insertion in plants through ZFN, MGN and TALEN techniques.

The findings of the patent search on targeted mutagenesis techniques in plants will be illustrated in the next paragraphs per single technique and finally as aggregated data.

Oligonucleotide directed mutagenesis (ODM)

Thirty-four patents have been identified on the use of ODM in plants. The distribution of these patents according to their priority date is illustrated in Figure 7.1. Priority date refers to the date of filing of the first member of each patent family (Parisi *et al.*, 2012). The first patent on ODM in plants was filed in 1991 (eight years before the first scientific publication) by the US-based company Berlex (Andrews *et al.*, 1991). The rest of patents have been filed between 1996 and 2010.

Most patents on ODM in plants have been filed by US-based assignees (Table 7.1), the rest by EU-based assignees, with the exception of one from Singapore. Out of the 34 patents identified, the majority (24) have been filed by industry and 10 by academic institutions. Industry is therefore clearly dominating in numbers of patents, however, when analysing the patents that receive the highest number of citations, the picture is different. As described in section 5.3.3 on methodology, key patents on ODM in plants were identified by looking at the most cited patents by the other members of the patent group. Out of the 34 patents identified on ODM in plants, the most cited are the following:

- Kmiec, E.B., 1996. Chimeric mutational vectors having non-natural nucleotides, WO/97/48714. **Thomas Jefferson University (US), University of Miami (US)** (cited by 9 other patents in the group)

- Kmiec, E.B., Gamper, H.B., Rice, M.C., 2000. Targeted chromosomal genomic alterations with modified single stranded oligonucleotides, EP/1268768. **University of Delaware (US)** (cited by 6 other patents in the group).
- Kmiec, E.B., Gamper, H.B., Rice, M.C., Kim, J., 2000. Targeted chromosomal genomic alterations in plants using modified single stranded oligonucleotides, US/2003/236208. **University of Delaware (US)** (cited by 6 other patents in the group).

Therefore, the key patents on ODM have been produced by the academic sector, in particular by three US-based universities. Additionally, as explained in section 5.3.3, the most cited patents by those outside the group of 34 on ODM in plants was analysed. In this case, the most cited patent is one claiming the use of ODM in mammalian and yeast cells, which was also produced by the academy:

- Kmiec, E.B., 1993. Compounds and methods for site directed mutations in eukaryotic cells. US/5565350. **Thomas Jefferson University (US)** (cited by 9 patents in the group)

What all these key patents have in common is one inventor. They were all produced by Eric B. Kmiec, who also co-authored the most important papers about ODM in plants (Gamper *et al.*, 2000; Rice *et al.*, 2000; Kmiec *et al.*, 2001) and might therefore be considered as one of the "parents" of ODM technique.

The University of Delaware, together with the companies Cibus and Pioneer Hi-Bred, appear in the findings of both patent and literature search (see section 6.2.1), showing that they are very active both in research and in industrial applications.

The information disclosed in patent claims is often complex in terms of legal language and structure, when compared to scientific papers (Parisi *et al.*, 2012). Different subjects can be identified in the claims of patents on ODM in plants,: usually the method (process) of targeted mutagenesis, the oligonucleotide used (tools) and the modified plant/seed or other organism obtained (products). By analysing the first, dominant claim of each patent, as explained in section 5.3.3, most patents on ODM in plants (17 out of 34) claim, as main subject, a method, while 11 patents claim a nucleic acid, usually the specific oligonucleotides employed, and only three patents have the modified plant as subject of the first claim.

Claims do not always make specific reference to the plants in which the method could be used and to the traits that could be obtained. For the patents in which this information was clearly available, the data were compared with results from scientific literature. As in the scientific literature, the main trait described for ODM in patents is herbicide tolerance. Some additional traits appear in patent claims and not in literature, in particular modification of fatty acid composition (Knuth *et al.*, 2006), fungal resistance (De Wit *et al.*, 2004) and prevention of dehiscence (Sundaresan and Rajani, 2000). Regarding plants, patents are generally broader in terms of plants listed, compared to literature, probably in order to maintain the field of

application of the invention as broad as possible, but is does not mean necessarily that the method have been tested in all plants claimed, unlike research papers. The plants mentioned the most in claims of ODM patents are maize and oilseed rape.

By analysing the official websites and press releases of the main companies emerging from the patent search on ODM in plants, additional information can often be found on the level of development of the techniques and potential products to be put on the market.

Cibus Company (www.cibus.com) developed ODM technique under the name of RTDS™ (Rapid Trait Development System) and produced an herbicide resistant oilseed rape, which is already tested in field trials in several States in the US and in the UK (http://www.defra.gov.uk/acre/files/20110319-Cibus-advice.pdf). According to the information in press releases, this oilseed rape will be soon commercialised by BASF. Additionally, Cibus seems to be developing several other crops with ODM in partnership with other organisations: modified flax with the Flax Council of Canada, a modified potato with the Company NEU Seed, oilseed rapes with new traits (related in particular to oil quality) with BrettYoung and other five major crops with a European focus with Makhteshim-Agan. Potatoes seem to be also in Cibus' pipeline with the trait of resistance to blackspot bruising.

The EU-based Company Keygene (www.keygene.com) also developed a version of ODM technique called KeyBase™. Examples of KeyBase mutants that have already been created according to Keygene's Website are herbicide-tolerant tobacco, tomato and Petunia plants.

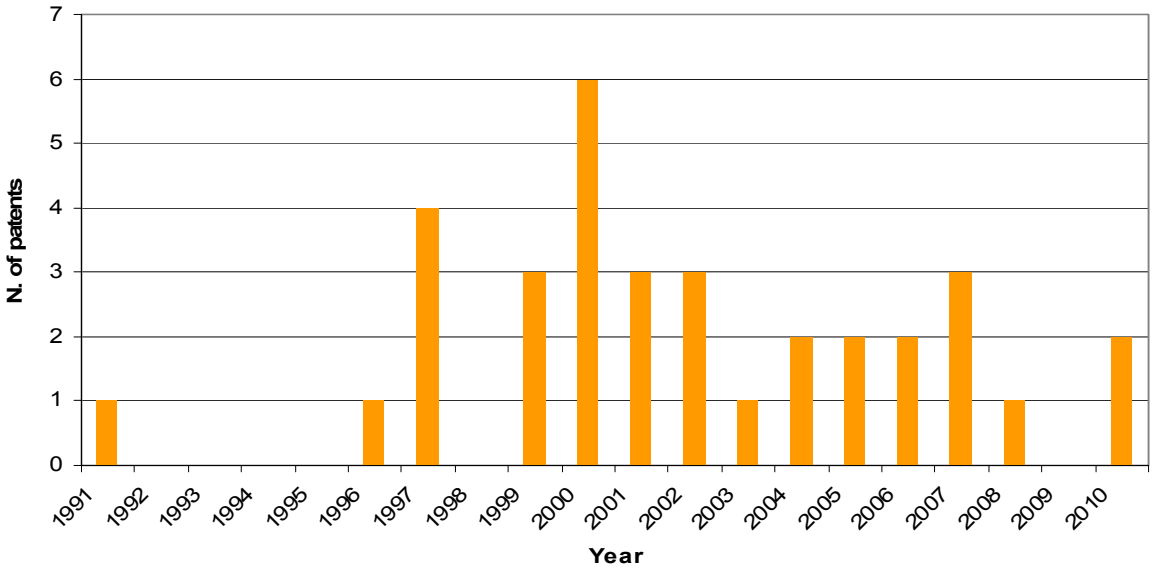

Figure 7.1 Development over time of patents on ODM in plants. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.1 Geographical distribution of patent assignees on ODM in plants. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	N. patents
EU-27	**10**
Netherlands	8
Germany	1
United Kingdom	1
North America	**25**
USA	25
Asia	**1**
Singapore	1

Zinc finger nuclease (ZFN) techniques (ZFN 1,2,3)

By including all three uses of the technique (ZFN 1,2,3) in the search, 31 patents have been identified on ZFN techniques in plants. As illustrated in Figure 7.2, all patents are concentrated in the last decade and reached a peak in 2009. As for ODM, most patents (27) on ZFN techniques have been filed by US-based institutions (Table 7.2) and the rest by institutions from the EU, Asia or rest of countries. Similarly to ODM, most patents on ZFN (25) are produced by industry, while only 3 are from academy and other 3 from joint collaboration between industry and academy.

The most cited patents within the group of patents on ZFN techniques in plants are the following:

- Miller, J.C., Zhang, L., 2004. Methods and compositions for targeted cleavage and recombination, WO/2005/084190. **Sangamo Bioscience Inc (US)** (cited by 6 other patents in the group)
- Liljedahl, M., Aspland, S.E., Segal, D.J., 2002. Methods and compositions for using Zinc Finger endonucleases to enhance homologous recombination, WO/03/080809. **Sangamo Biosciences Inc (US)** (cited by 6 other patents in the group).
- Carroll, D., Bibikova, M., Drews, G.N., Golic, K.G., Golic, M.M., 2002. Targeted chromosomal mutagenesis using Zinc Finger nucleases. WO/2003/087341. **University of Utah Research Foundation (US)** (cited by 5 other patents in the group).

Two key patents on ZFN techniques in plants have been filed by the same US-based company, Sangamo Biosciences, which also possess the highest number of patents in the field (14 out of 31), and one by the University of Utah, also US-based. By analysing the number of citations by patents outside the group of 31 identified, a key patent on the use of ZFN techniques in human and animal cells clearly emerges:

- Holmes, M.C., Urnov, F., Gregory, P.D., Rebar, E.J., Brennan, S.M., 2005. Targeted integration and expression of exogenous nucleic acid sequences. WO/2007/014275. **Sangamo Biosciences Inc (US)** (cited by 10 patents in the group)

Again Sangamo Biosciences confirms to be a key actor in the field of ZFN techniques. This company filed indeed tens of patents on ZFN techniques in several organisms.

Several institutions seem to be dealing with ZFN techniques and there is a quite big overlap between literature authors and patent assignees, both from industry and academy. In particular, the following institutions appear in both sources: from industry Sangamo Biosciences (US), Dow Agrosciences (US), Danziger Innovations (IL) and Toolgen (KR); from academy University of Utah Research Foundation (US), the Hebrew University of Jerusalem (IL), the National Institute of Agrobiological Sciences (JP) and Seoul National University (KR).

The subjects identified in the claims of patents on ZFN techniques in plants are usually the method (process) of targeted mutagenesis, the nucleases employed, including the nucleic acid sequence econding them and the vector used for delivery them to the host organisms, (tools) and the modified plant/seed or other organism obtained (products). By analysing the first, dominant claim of each patent, as explained in section 5.3.3, most patents on ZFN techniques in plants (18 out of 31) claim, as main subject, a method, while nine patents claim the chimeric nuclease employed and only one patent have the modified organism as subject of the first claim.

Regarding traits claimed in patents on ZFN techniques in plants, most traits overlap with information derived from scientific literature, in particular herbicide tolerance and reduced levels of phytic acids. Additional traits that appears in patent claims are male sterility (Vainstein and Zuker, 2008, 2009) and production of pharmaceuticals in plants (Carroll *et al.*, 2002). Regarding plants, only few patents claim a specific crop plant, in particular maize (Butler *et al.*, 2009).

From scientific literature and patent search it emerges that Sangamo (www.sangamo.com) is the key player in the field of ZFN techniques. According to the information contained in its Website and press releases, Sangamo created a method for using ZFNs for targeted mutagenesis and integration called EXZACT™ (www.exzactprecisiontechnology.com) and conferred exclusive rights to the Company Dow AgroSciences (www.dowagro.com) for its use in agricultural biotechnology. According to its Website, Dow Agrosciences is developing the techniques in maize for herbicide tolerance, in Sugar beet together with KWS Company, in potato for starch quality together with Wageningen University and in trees for lumber and paper production together with Oregon State University.

The Companies Danziger Innovations (www.danziger-innovations.com) and Toolgen (www.toolgen.com) also developed methods for targeted mutagenesis through ZFNs and called

them MemoGene™ and GeneGrip®, respectively. In its Website, Danziger Innovations declares that MemoGene™ technique was validated in tobacco, tomato, Petunia, *Arabidopsis*, lettuce, *Agyranthemum* and *Populus tremula* but no potential commercial products are revealed. Toolgen only declares to have tested GeneGrip® in *Arabidopsis*.

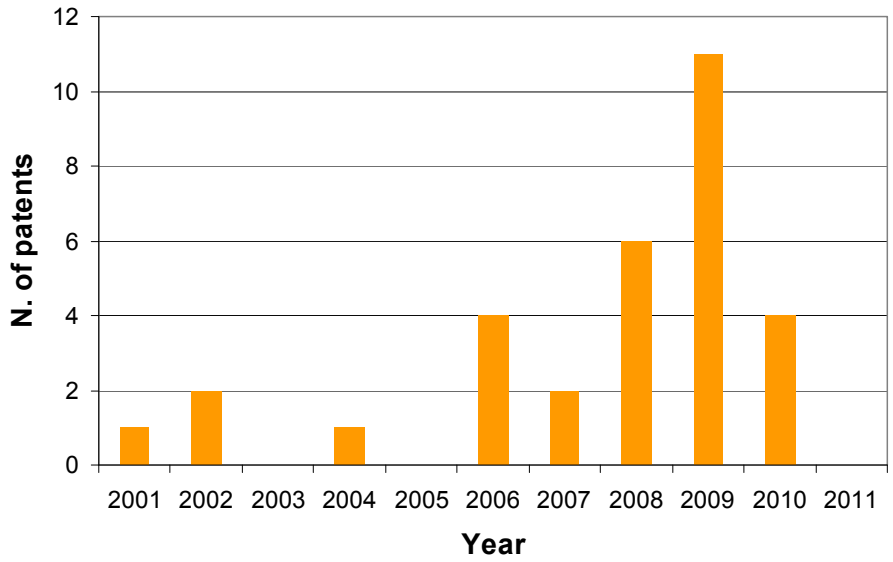

Figure 7.2 Development over time of patents on ZFN in plants. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.2 Geographical distribution of patent assignees on ZFN techniques in plants. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	N. patents
EU-27	**6**
Germany	2
Netherlands	2
France	1
Belgium	1
North America	**27**
USA	27
Asia	**5**
South Korea	3
China	1
Japan	1
Other countries	**4**
Israel	4

Meganuclease (MGN) techniques (MGN1,2,3)

Despite the very low number of scientific publications compared to ODM or ZFN techniques, already 29 patents have been identified on the use of MGN techniques in plants. The first patent identified was filed in 1994 by the Institute Pasteur (FR) on the use of the MGN Sce-I to produce a DSB in its recognition site in plant cells. Eight years after this initial patent, more methods of MGN use in plants have been patented (Figure 7.3). Unlike ODM and ZFN techniques most patents on MGN in plants have been produced by EU-based institutions (Table 7.3), in particular Cellectis Company, based in France, that filed several patents on MGN applied to several organisms and 17 for applications in plants. The majority of patents identified (27) have been filed by industry and only two by academy.

Within the group of patents identified on MGN techniques in plants, the most cited ones are the following two, both filed by Cellectis:

- Arnould, S., Chames, P., Choulika, A., Epinat, J.C., Lacroix, E., 2002. Hybrid and single chain meganucleases and use thereof. WO/03/078619. **Cellectis (FR)** (cited by 6 other patents in the group)
- Arnould, S., Bruneau, S., Cabaniols, J.P., Chames, P., Choulika, A., Duchateau, P., Epinat, J.C., Gouble, A., Lacroix, E., Paques, F., Perez-Michaut, C., Smith, J., Sourdive, D., 2003. Custom-made meganuclease and use thereof. WO/2004/067736. **Cellectis (FR)** (cited by 5 other patents in the group).

By analysing the number of citations by patents outside the group on plants, the following key patents emerge, all about the use of MGNs in human and animal cells for therapeutic reasons: treatment of genetic disorders, infections or cancer:

- Arnould, S., Perez-Michaut, C., Smith, J., 2006. Meganuclease variants cleaving a DNA target sequence from a Xeroderma Pigmentosum gene and uses thereof. WO/2007/093918. **Cellectis (FR)** (cited by 5 patents in the group)
- Hellinga, H., Smith, J.J., Jantz, D., 2005. Rationally-designed meganucleases with altered sequence specificity and DNA-binding affinity. WO/2007/047859. **Precision Biosciences (US)** (cited by 5 patents in the group)
- Stoddard, B.L., Monnat, R.J.J., Baker, D., Chevalier, B., Kortemme, T., Chadsey, M., 2002. Methods and compositions concerning designed highly-specific nucleic acid binding proteins. WO/2004/031346. **Fred Hutchinson Cancer Research Center (US)** (cited by 5 patents in the group).

Cellectis is still present in the list of key patents, together with two institutions from the US.

Four private companies have produced both scientific papers and patents about the use of MGNs in plants for targeted mutagenesis or gene insertion, three from the US: Pioneer Hi Bred, Precision Biosciences and Biogemma, and one from the UE (Belgium): Bayer Bioscience.

Similarly to ZFN techniques, the subjects identified in claim of patents on MGN techniques are usually the method (process) of targeted mutagenesis, the nucleases used, including the nucleic acid sequence econding them and the vectors used for delivery them to the host organisms, (tools) and the modified plant/seed or other organism obtained (products). By analysing the first, dominant claim of each patent, as explained in section 5.3.3, most patents on MGN techniques in plants (16 out of 29) claim, as main subject, a method, while 11 patents claim the meganuclease employed and two patents the nucleic acid sequences of the meganucleases.

Most patents identified on MGN techniques in plants show very generic claims in terms of plants and traits. As in scientific literature, the only crop plant claimed is maize (D'Halluin *et al.*, 2003; Jantz and Smith, 2008) and no new traits emerge compared to scientific literature, despite the high number of patents. Only one patent with broad claims (D'Halluin, 2011) mentions the possibility of using MGN techniques for obtaining resistance against biotic and abiotic stress.

Also according to the information contained in their Websites, Cellectis (www.cellectis.com) and Precision BioSciences (www.precisionbiosciences.com) design MGNs for targeted gene modifications. Cellectis Plant Sciences gave non-exclusive license to Monsanto for the use of MGN techniques in crops, but no specific products are described in the Web. Precision BioSciences gave the license about its MGN technique, called DNE (Directed Nuclease Editor), to Dupont and BASF and, according to its Website, the technique has already been validated in multiple research and commercial crop species including *Arabidopsis*, tobacco and maize amongst others.

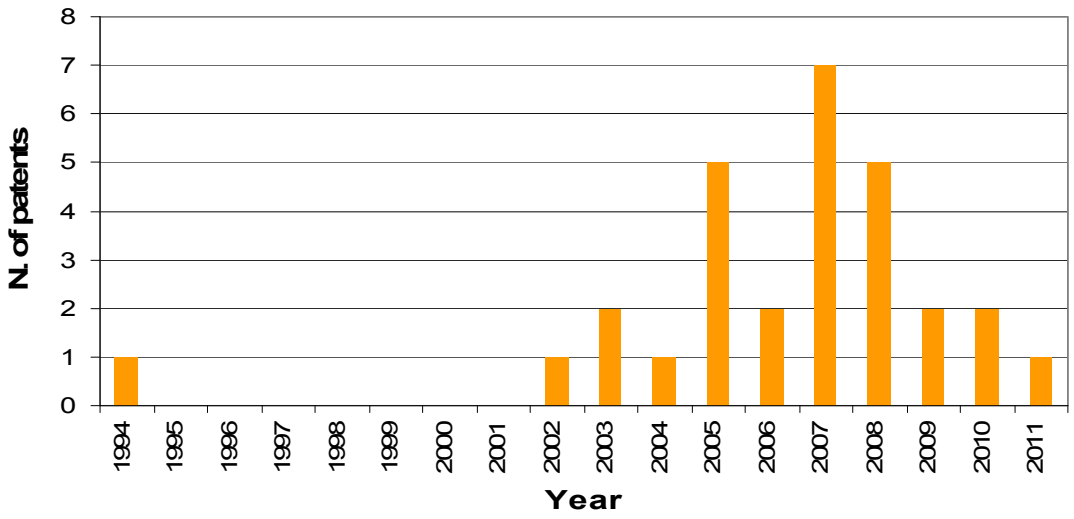

Figure 7.3 Development over time of patents on MGN in plants. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.3 Geographical distribution of patent assignees on MGN techniques in plants. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	N. patents
EU-27	**27**
Germany	1
Belgium	4
France	22
North America	**4**
USA	4
Asia	**1**
China	1

Transcription Activator-Like Effector Nuclease (TALEN) techniques

Due to the novelty of techniques, only five and very recent patents have been identified, as represented in Table 7.4 The five patents include also a patent in which only TAL effectors are used to target specific DNA sequences in plants and modulate their expression without introducing any nuclease (Boch *et al.*, 2009) This patent was filed by inventors not affiliated to any institutions and coming from Belgium and Germany. As shown in Table 7.4, three other patents are from academic institutions of the US and Germany, while the fifth patent was produced by a joint collaboration between a University and a Company in South Korea.

Only one institution, the University of Minnesota, has been active both in scientific literature (with two publications) and in patents on TALEN techniques in plants.

Due to the low number of patents, citation analysis is not very meaningful and indeed there are no common citations among the five patents identified. From claims analysis, similarly to ZFN and MGN techniques, the method (process) of targeted recognition and mutagenesis, the nucleases and TAL-effectors used, including the nucleic acid sequence econding them and the vectors used for delivery them to the host organisms, (tools) and the modified organisms obtained (products). By analysing the first, dominant claim of each patent, as explained in section 5.3.3, three patents claim, as main subject, the method, while the other two claim the chimeric nucleases.

The claims do not make reference to specific plants or traits. Patents produced in the future might reveal more specific use of the method and probably cite these first ones as key patents with broader claims.

According to the information available in the Web, the two key institutions in the developments of TALEN techniques are Cellectis and Two Blades Foundation. However, due to the the very recent discovery of the technique, no specific information is revealed about potential products obtainable in the agricultural sector.

Table 7.4 Patents identified on TALEN techniques in plants.

Patent	Year	Country	Sector	Institution
Boch *et al.* (2009)	2009	DE, GB	industry	Private inventors
Voytas *et al.* (2009)	2009	US	academy	University of Minnesota
		US	academy	University of Iowa Research Foundation
Kuehn *et al.* (2010)	2010	DE	academy	Helmholtz Zentrum Muenchen
Yang *et al.* (2010)	2010	US	academy	University of Iowa Research Foundation
Kim and Kim (2011)	2011	KR	industry	Toolgen Inc
		KR	academy	Seoul National University R&DB Foundation

Targeted mutagenesis – aggregated data

Patents identified on the four techniques analysed in this section have been aggregated for a global analysis. A shown in Figure 7.4 the 99 patents indentified in total on targeted mutagenesis and targeted integration in plants are mostly concentrated in the last decade. A growing trend in number of patents can be observed until 2009. The data for 2010 and 2011 could be incomplete because there if often a delay between priority date and date of publication, therefore the number of patents could be still growing but it is too early to evaluate.

US-based institutions have the lead in patents on targeted mutagenesis techniques (same as in scientific publications), but are followed closely by the EU (Table 7.5). Industry is dominating with 77 patents, while 18 have been produced by academy and only four by joint collaborations.

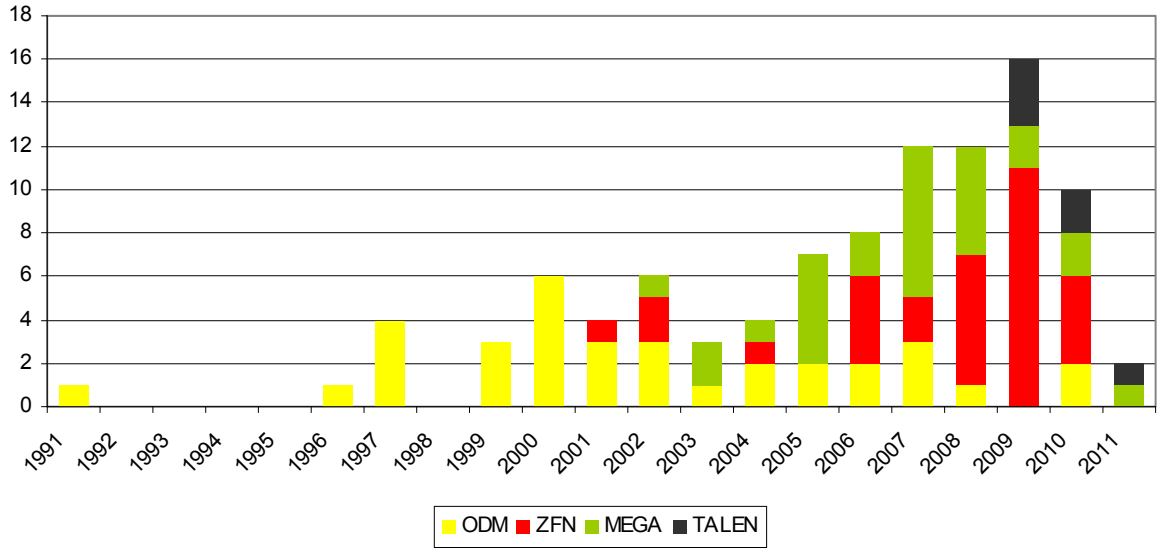

Figure 7.4 Development over time of patents on targeted mutagenesis and insertion in plants. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.5 Geographical distribution of patent assignees on targeted mutagenesis and insertion in plants. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	**N. patents**
EU-27	**45**
France	23
Netherlands	10
Germany	6
Belgium	5
United Kingdom	1
North America	**59**
USA	59
Asia	**9**
South Korea	5
China	2
Singapour	1
Japan	1
Other countries	**4**
Israel	4

7.2.2 Techniques resulting in "Negative segregants": Reverse breeding, RdDM, Early flowering

RNA-dependent DNA methylation (RdDM)

There are several patents on plants mentioning the mechanism of RdDM in their description and sometimes even in claims, but only one patent has been identified that reflects the scope of the new technique RdDM as defined in this study: the induction of transcriptional gene silencing in a plant through a transgenesis step with the subsequent removal of the transgene. The patent identified (Table 7.6) was filed in 2008 by the German private Company Rlp Agroscience GmbH, which also produced a publication in the topic.

The patent identified has mixed claims, on the process and the plant products obtainable, however, the first dominant claim (see section 5.3.3) referred to the method of RdDM. No specific plant species are claimed in the patent identified. The examples of genes that could be silenced, according to claims, are: genes encoding a product that is harmful for animals, humans or plants, like genes encoding allergens or genes influencing the level of poisonous biochemical substances in a plant and genes encoding an unwanted trait as for example a gene involved in the onset of over-ripeness.

Table 7.6 Patent identified on RdDM in plants.

Patent	Year	Country	Sector	Institution
Wassenegger *et al.* (2008)	2008	DE	Industry	Rlp Agroscience GmbH

Reverse Breeding

Two patents were identified on Reverse Breeding (Table 7.7), filed by the Dutch private Company Rijk Zwaan in 2001 and 2005. Rijk Zwaan produced also a key publication in 2009 (Dirks *et al.*, 2009).

Both patent identified have mixed claims, referring to the method of Reverse Breeding and the plant products obtainable, however, by analysing only the the first dominant claim (see section 5.3.3), the main subjects of claims is the method. In both patents, the invention is claimed for plants in general, without mentioning specific plant species. Since the objective of the invention is to make parental lines for the production of F1 hybrid seed, no specific traits are described.

Table 7.7 Patent identified on Reverse Breeding.

Patent	Year	Country	Sector	Institution
Dirks *et al.* (2001)	2001	NL	industry	Rijk Zwaan Zaadteelt en Zaadha
Van Dun and Dirks (2005)	2005	NL	Industry	Rijk Zwaan Zaadteelt en Zaadha

Accelerated breeding through induction of early flowering

Countless patents related to the mechanisms of early-flowering have been produced and it is a complex task to distinguish between the analysis of the potential of genes that confer early flowering trait and the intention of inducing early flowering in intermediate generations in the breeding process, to finally select only negative segregants. Therefore, the patent search was not carried out for this technique.

However, according to the information extracted from the Web, some products are already being developed with commercial intention. In particular, the USDA-ARS Appalachian Fruit Research Station in the US developed an early-flowering construct and applied it in plum to lessen the time it takes to create new varieties (http://www.ars.usda.gov/is/ar/archive/mar11/breeding0311.htm?pf=1) without having any transgenes in the final products.

7.2.3 Variants of plant transformation techniques: Cisgenesis, Intragenesis and Grafting non-GM varieties onto GM rootstock

Cisgenesis and Intragenesis

As for the scientific literature search (see section 6.2.3), Cisgenesis and Intragenesis techniques were grouped together in the same patent search, since their definitions are often overlapping. Twenty patents have been identified in total on the two techniques, with a peak in 2007 (Figure 7.5). As shown in Table 7.8, most patents were produced by US or EU-based (especially from the Netherlands) assignees, in particular in industry (15 patents), while only three patents are from academy and two from joint collaborations.

The most cited patent within the group of patents on cisgenesis/intragenesis in plants is the following, produced by the US Company Simplot, which owns seven of the 20 patents identified on cisgenesis/intragenesis and ten publications:

- Rommens, C.M.T., Ye, J., Yan, H., Swords, K.M.M., Menendez-Humara, J., Brinkerhoff, L.W., Richael, C., Weeks, J.T., 2002. Precise breeding, WO/03/069980. **J.R. Simplot Company (US)** (cited by 5 patents in the group).

The patent is about a process to obtain plants (like potato) that only contain nucleic acid from the plant species selected for transformation or from plants that are sexually compatible. The main traits claimed are reduced black spot bruising and reduced cold-induced sweetening.

No relevant citations have been identified outside the group of the 20 patents on cisgenesis and intragenesis.

Besides Simplot Company, other institutions that have been active both in scientific literature and in patents on cisgenesis/intragenesis are Wageningen University in the Netherlands and the New Zealand Institute for plant and food research Limited.

By analysing claims, the main subjects identified of the patents on cisgenesis and intragenesis are the method (process) of plant transformation, the sequences and vectors used (tools) and the plant/seed obtained (products). Regarding the analysis of the first, dominant claim (see section 5.3.3), nine patents claim the method as main subject, eight claim the sequence used for transformation and three the vector or cassette used.

The plant which is most widely mentioned in claims (in 15 out of 20 patents) is potato. Therefore, most identified traits refer to potato and are generally overlapping with traits reported in literature: fungal resistance (usually against *P.infestans*), lower levels of acrylamide and reduced black- spot bruising. New traits reported in patents compared to literature are reduced cold-induced sweetening (Rommens *et al.*, 2002; Richael, 2006), resistance to virus (Jahn and Cavatorta, 2008; Rommens *et al.*, 2010) and nematodes (Van der Vossen *et al.*, 1998) and modified levels of flavonoids and anthocyanins (Luo *et al.*, 2008).

Simplot Company (www.simplot.com) trademarked its intragenesis method under the name of Innate™ and the information on its Website confirms the developments of potatoes with reduced black spot bruise, lower asparagine and slower degradation of starch to sugar during storage (thereby reducing acrylamide content). Multiple filed trials were conducted over 2009 and 2010 in several States.

Wageningen University, according to the Website dedicated to cisgenesis (www.cisgenesis.com), developed cisgenic potatoes resistant to late blight, already in field trials as illustrated in section 6.2.3, but also potatoes resistant to nematodes and with high carotenoids content, still in research phase. Additionally, Wageningen University developed cisgenic apples resistant to apple scab (also in field trials, see section 6.2.3) and apples with red flesh.

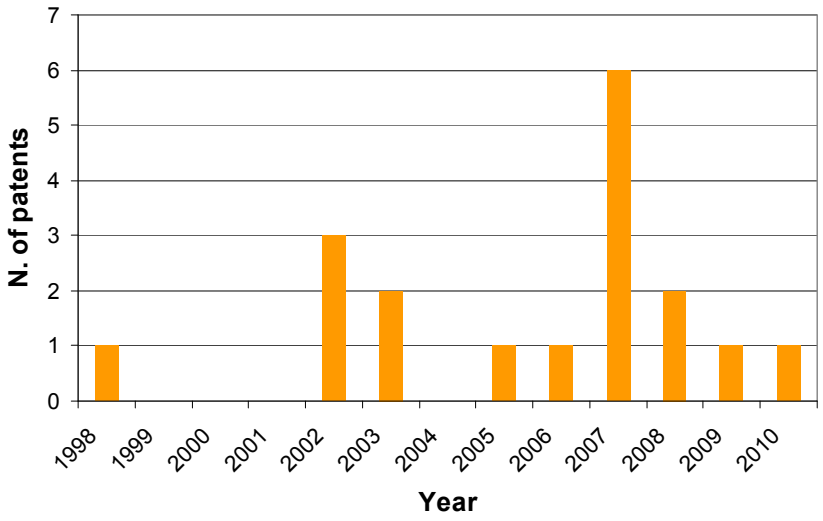

Figure 7.5 Development over time of patents on cisgenesis and intragenesis in plants. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.8 Geographical distribution of patent assignees on cisgenesis and intragenesis in plants. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	N. patents
EU-27	**10**
United Kingdom	2
Netherlands	8
North America	**10**
USA	10
Other countries	**2**
New Zealand	2

Grafting non-GM varieties onto GM rootstocks

Fifteen patents have been identified on the development of GM rootstocks to be used for grafting of non GM scions. Those patents have been filed between 1992 and 2009 (Figure 7.6) by institutions from different countries, in particular the US (Table 7.9). Nine patents have been filed by industry and six by academy. The 15 patents are quite heterogeneous and indeed no common citations have been identified within the group. They all generally cite older patents on transformation techniques, which vary also in relation to the specific plant transformed.

By analysing claims, the main subjects identified of the patents on grafting on GM rootstock are the method (process) of plant transformation, the sequences and vectors used (tools) and the plants and rootstocks obtained (products). Regarding the analysis of the first, dominant claim (see section 5.3.3), five patents claim the sequence used for transformation or the protein obtained as main subject, four the method of plant transformation and four claim the plant obtained.

Most of the patents identified claim specifically certain crop plants for the obtainment of specific traits. Compared to scientific literature data, some plant/trait combinations are confirmed, like virus resistance in grapevine (Gonsalves and Meng, 1997; Gonsalves *et al.*, 1997; Zhu *et al.*, 1997) and cucumber (Gal-on *et al.*, 2004) and new possibilities also emerge from claims, like fungal resistance in apple and pear (Aldwinckle and Norelli, 1992), salt resistance in apple (Shenchun, 2006; Fengwang *et al.*, 2009) and virus resistance in tomato (Polston and Hiebert, 2004) and citrus (Gmitter *et al.*, 2001).

Two academic institutions have been active in this technique by producing both papers and patents: Clemson University Research Foundation from the US and the Northwest A&F University from China.

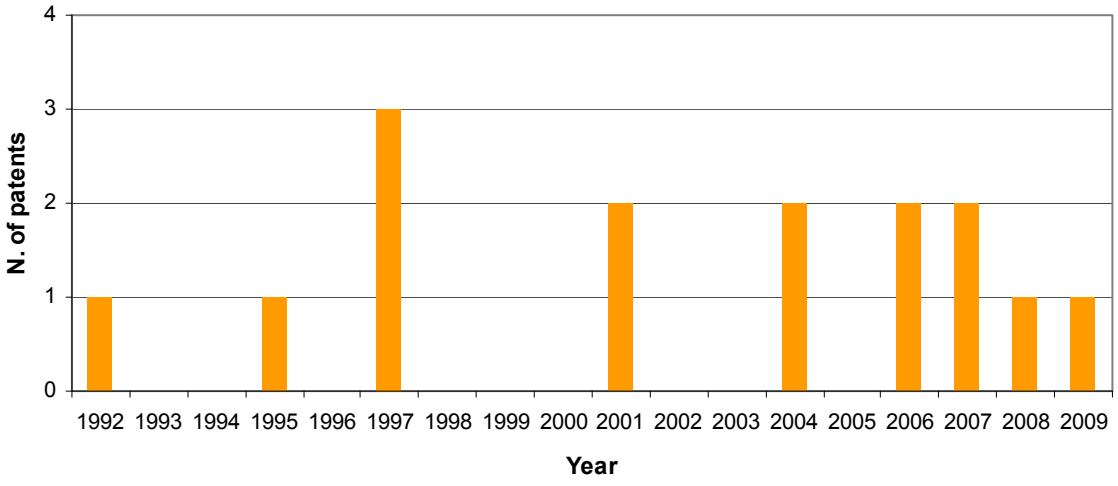

Figure 7.6 Development over time of patents on grafting on GM rootstock. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.9 Geographical distribution of patent assignees on grafting on GM rootstock. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	N. patents
EU-27	**1**
Germany	1
North America	**10**
USA	10
Asia	**2**
China	2
Other countries	**2**
Israel	2

7.2.4 Aggregated results

In total, 137 patents have been retrieved on NPBTs in the search. Their distribution per year (Figure 7.7) reveals a growing trend, considering that the data for 2010 and 2011 are still incomplete. As shown in the figure, the first patents to be produced were for ODM and grafting on GM rootstocks, while TALEN technique is clearly the most recent in being developed, as already seen in literature production.

Regarding assignees of patent on NPBTs (Table 7.10 and Figure 7.8), most of them (more than 50%) are from the US, followed closely by EU-based assignees with a 37.6% of patents, produced especially by France and the Netherlands. Out of the 137 patents, three fourth (104 patents) have been produced in the industry, while one fifth (27 patents) are from academy and only 6 patents from joint collaboration between an academic and a private institutions.

The leading institutions on patenting activity in the field of NPBTs were identified by analysing all assignees of the patents identified. In total 53 institutions are active in patenting on NPBTs, less than one third of the ones emerging in the literature search. Considering the total number of patents filed, the French Company Cellectis is in leading position (Table 7.11). The only public institution appearing in the top 10 list in terms of number of patents is the University of Delaware, from the US. Compared with the most active institutions in literature, here it can be observed that in most cases institutions are specialised in one or two techniques. The only exception (not only in the top ten but also screening all the 53 institutions) is the multinational Company BASF, active in three techniques, all for targeted mutagenesis: ODM, ZFN and MGN techniques.

Figure 7.9 shows the distribution of patent applications to the USPTO and the EPO, and additionally the patent applications that went through the PCT route and are administered by

WIPO. PCT is a route followed to obtain protection in any or all contracting states (see section 3.4.1). Within 18 months of the PCT application, the inventor can select the patent offices of the countries in which to protect the invention, including the EPO, the USPTO and other national offices. Therefore, the same application can be submitted to several offices and this explains the overlaps in number of patents shown in Figure 7.9. The patent search shows that most applications (96%) are found in the WIPO database, meaning that applicants followed the PCT route. The percentage of patents submitted to the USPTO (85% of the total) and to the EPO (73% of the total) follows closely, suggesting that applicants see commercial interest in both the European and North American markets. Figure 7.9 also reports the data on the national patent offices in which patents on NPBTs have been filed, demonstrating that the markets with the greatest interest after US and EU are, in this order, Canada (65% of patents), Australia (64%), Japan (40%) and China (38%).

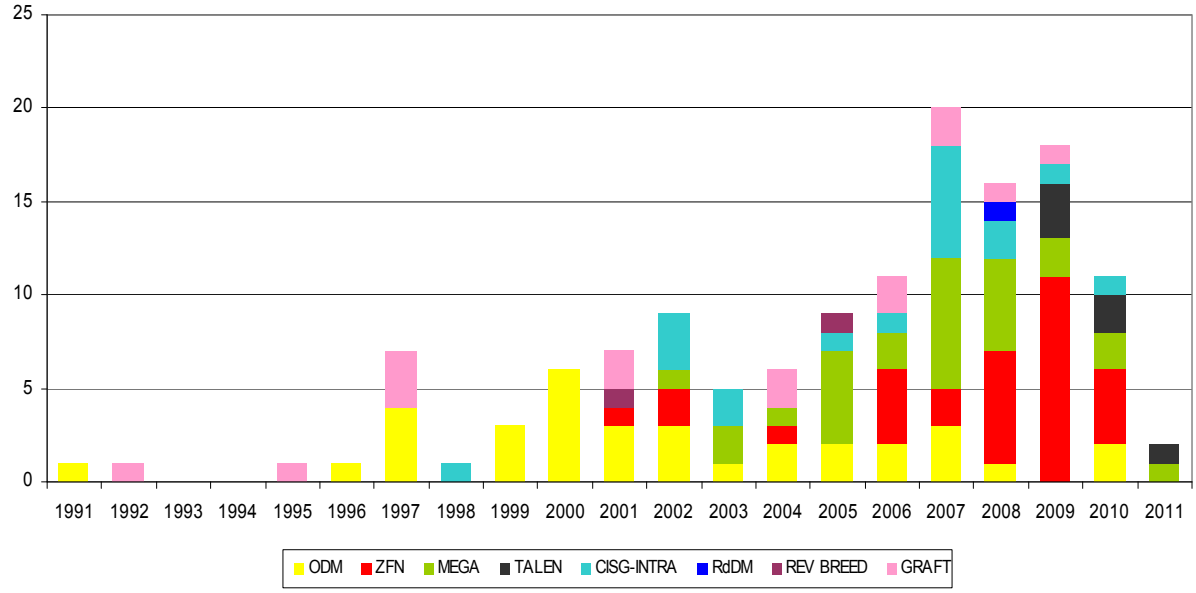

Figure 7.7 Development over time of patents on NPBTs. Priority date (date of first application) of each patent is given on the x axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 7.10 Geographical distribution of patent assignees on NPBTs. Each number refers to the number of patents with at least one assignee from the specific country.

Authors country	N. patents
EU-27	**59**
France	23
Netherlands	20
Germany	8
Belgium	5
United Kingdom	3
North America	**79**
USA	79
Asia	**11**
South Korea	5
China	4
Singapour	1
Japan	1
Other countries	**8**
Israel	6
New Zealand	2

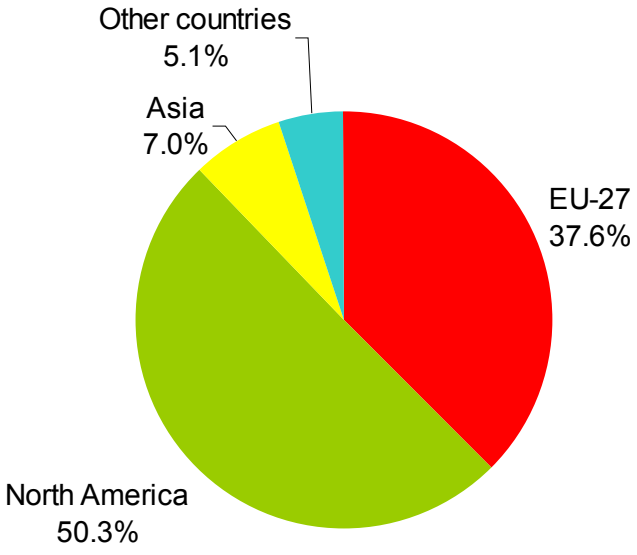

Figure 7.8 Geographical distribution of patent assignees on NPBTs.

Table 7.11 Most active institutions in NPBTs in plants, based on number of patents produced (second column from the right) and number of techniques covered (first column on the right). Each technique is represented by a letter. O:ODM, Z:ZFN, M:MGN, T:TALEN, C: Cisgenesis/Intragenesis, R:RdDM, B:Reverse Breeding, G:Grafting.

Institution	Sector	Country	N. patents	Techniques
Cellectis	**Industry**	FR	17	M
Sangamo Biosciences	**Industry**	US	13	Z
Dow Agrosciences	**Industry**	US	11	Z
Keygene	**Industry**	NL	8	O,Z
J.R. Simplot	**Industry**	US	7	C
University of Delaware	Academy	US	7	O
BASF	**Industry**	DE, NL, CN	6	O,Z,M
Bayer	**Industry**	BE, FR	6	Z,M
Cornell Research Foundation	**Industry**	US	5	G
Pioneer Hi Bred	**Industry**	US	4	O,M

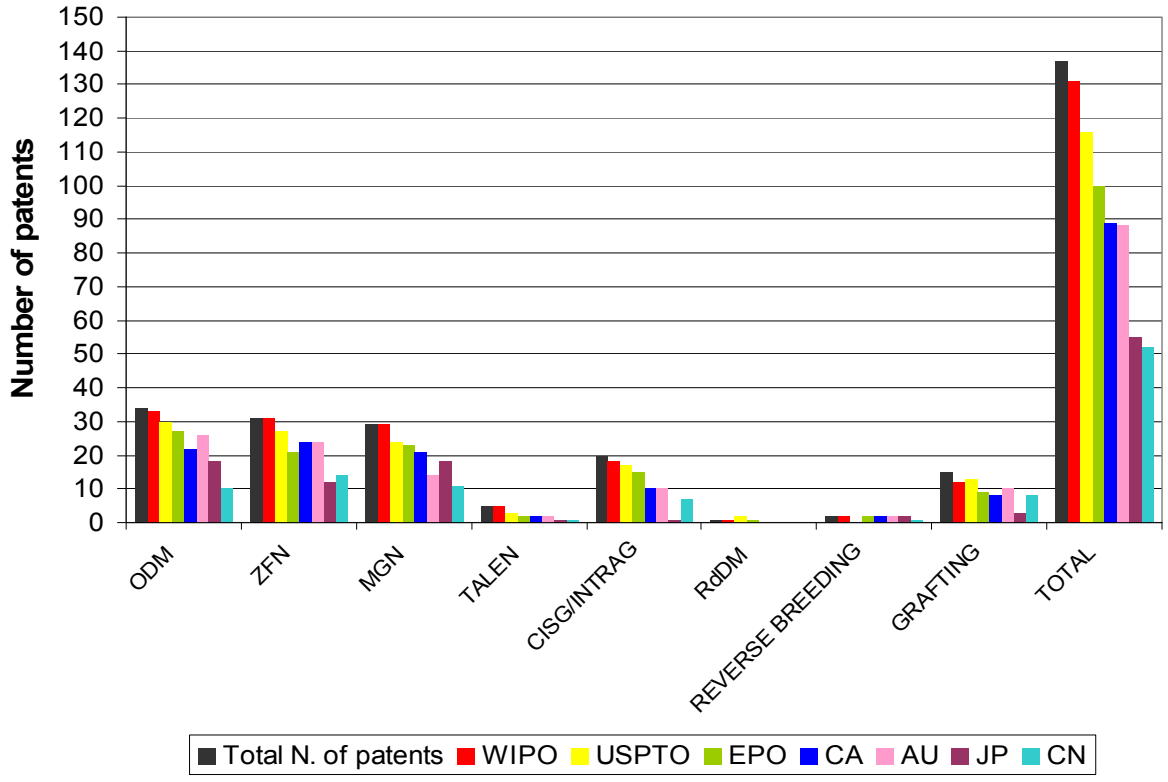

Figure 7.9 Patents on NPBTs filed at different patent offices and PCT applications (administered by WIPO), distributed per technique. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family. Each patent office is represented by an acronym. USPTO: United States Patent and Trademark Office, EPO:European Patent Office, CA:Canada, AU:Australia, JP:Japan, CN:China.

7.3 Conclusions

Patent landscape analysis confirmed that the field of NPBTs is very active, as observed with scientific literature results, being most patents produced in the last decade, with the exception of few initial patents on ODM, cisgenesis and grafting on GM rootstock filed before the year 2000.

Regarding the amount of patents identified per technique compared to the literature results, some techniques revealed to be of potential interest both for research and for industrial applications, while other techniques show a different profile. The highest number of patents was identified for ODM and ZFN techniques, which are also subject of a relatively high number of papers considering that they are relatively young techniques and that its application in plants is a restricted field. They are followed by MGN, another targeted mutagenesis techniques, for which less papers have been produced so far, maybe due to the fact that the main organisation dealing with the technique gave priority to patenting than publishing. To complete the group of targeted mutagenesis techniques, TALEN techniques confirm their novelty in its application to plants and very few patents and papers have been produced so far, but higher numbers are foreseen for the near future. Globally, techniques for targeted mutagenesis seem to have a high potential since both literature and patent data are growing.

Cisgenesis, intragenesis and grafting on GM rootstock showed a high potential in research, being subjects of many papers. However, the number of patents on these techniques is smaller compared to the trend of targeted mutagenesis techniques. This may be due to the fact that the method employed (plant transformation) does not offer real novelty compared to pre-existing patents.

Reverse Breeding confirmed to be a recent technique in the hands of a Dutch institution. Papers and patents production is still scarce and no clear information is available yet about potential products. Finally, RdDM shows a very big potential for research, especially focused on the understanding of the mechanisms behind transcriptional gene silencing, however, the industrial interest seems to be very low, probably because the maintenance of the new traits is not guaranteed through generation and therefore industry does not see the possibility for concrete applications.

The highest number of patents in the field of NPBTs was produced by US-based institutions, followed by the EU. This is in contrast with the data from literature that shows the opposite scenario. In particular, US is leading in number of patents for ODM, ZFN techniques and grafting on GM rootstock, while EU is leading in MGN techniques, RdDM and Reverse Breeding. For TALEN and cisgenesis and intragenesis the same number of patents was filed by both the US and the EU.

Fewer institutions are active in patenting in the field of NPBTs compared to scientific literature and most of them, as expected, belong to the private sector. Most of the academic institutions patenting on NPBTs are US Universities. The stronger habit of patenting in the US, even in academic institutions, compared to the EU, could be a reason on the basis of the higher number of US patents, considering that research activities seem to be stronger in the EU.

Patent citation analysis offered different results depending on the specific technique. ODM seems to be fragmented among different institutions and to be characterised by a key role of the public sector in developing the technique. For ZFN and MGN it was confirmed the key role of a one specific Company in developing the technique (Cellectis and Sangamo, resectively). In ZFN academy seems to have partly contributed. TALEN is too recent to make a conclusive analysis. In cisgenesis and intragenesis a Company (Simplot) seems to be leading part of the sector, but different actors are involved. RdDM and Reverse Breeding have been patented so far by just one institution each and, finally, grafting on GM rootstock offers a heterogeneous field in terms of assignees and patents cited.

It can be observed that most patents on NPBTs have mixed claims, that include the process, the tools employed (proteins, sequences, vectors, etc.) and the final products (plants and other organisms). By analysing the first claim of each patent, which usually dominates on the other claims, for most techniques, there is prevalence on patents on methods (processes). This prevalence is less evident for patents on cisgenesis, intragenesis and grafting on GM rootstock, in which the method used of plant transformation is not novel, compared to the protocols of the other six techniques, which can be considered as the real "new" techniques.

From claims analysis plants and traits emerged in the literature as potential products of NPBTs have been largely confirmed by patent data, with the exception of some new technical possibilities described in patents and emerged by the analysis of main Companies' Websites. However, it is confirmed that main traits achieved through NPBTs are still herbicide tolerance and disease resistance.

The geographic coverage of patents identified is very large and spread towards all continents demonstrating that plant breeding industry sees a big potential worldwide for NPBTs.

Chapter 8. Results: Comparative analysis of NPBTs through case-studies

8.1 Introduction

Technical advances in plant breeding are of fundamental importance for breeders. They allow them to improve the process of obtaining new varieties by making it more efficient, faster or cheaper, or by enlarging the range of options for plant genome modification. However, all these improvements are not always reached with the same technique: Often a new technological option results more efficient and less time consuming but much more expensive. For example, it might require a bigger investment of money due to the need of more advanced equipment, of licenses of specific patents or because of regulatory costs, like in the example of transgenesis (see section 2.2.4). Therefore, plant breeders need to analyse the cost-effectiveness of new technologies before to incorporate them into their breeding programs (Brennan and Martin, 2007).

According to our findings in literature and patent search on NPBTs (chapters 6 and 7), several companies and academic institutes are currently researching alternatives to conventional plant breeding methods by making use of biotechnology. They may be motivated by the search of better cost-effectiveness, technical advantages or more options in exploiting plant germplasm. Their intent might also be related to the hope of finding a convenient alternative to transgenesis since the high regulatory costs of compliance with GMO regulation are not affordable by small and medium enterprises and academic institutions. An analysis of all factors involved would help identifying the main reasons of this growing trend towards new technologies.

In scientific literature it is common to find information on costs of plant breeding programs focused on farming activities, i.e. costs of plant treatments, fuel, supplies, labour in field etc. (Dreher *et al.*, 2003; Mangione *et al.*, 2006; Zangeneh *et al.*, 2010) and on comparison between different breeding programmes. However, there are not many publications available about costs related to the previous phase of obtaining the plant variety before to be released in the field.

Due to the novelty of NPBTs, information about adoption costs and costs related to field activities is not yet available and private companies prefer not to reveal data on field trials before to commercialisation of their products. Therefore, an analysis of cost-effectiveness of those techniques cannot be based on this phase due to absence of empirical data, but can be focused on laboratory procedures of plant breeding to obtain the desired plant variety.

According to literature, several researchers have analysed the cost-effectiveness and benefits of the incorporation of modern techniques onto breeding programmes, but most of those papers are focused on MAS or other selection techniques (e.g. plant physiological testing). The authors usually compare costs, time and efficiency of modern selection compared to conventional

methods based on phenotype observation. Regarding methodology, some papers report a global estimation of the cost of the entire selection process to be compared with the conventional one, without comparing the costs associated to the single steps of the technological process (Moreau *et al.*, 2000; Concibido *et al.*, 2004; Kuchel *et al.*, 2005; Bernardo and Yu, 2007; Wong and Bernardo, 2008). Some other papers provide a detailed description of the single steps taken into account for the cost calculation (Dreher *et al.*, 2003; Bagge and Lubberstedt, 2008).

According the latter, the overall conclusion is that MAS is usually faster than phenotypic selection, while its cost-convenience is mostly related to the specific application: according to Kuchel et al (2005), the economic success of MAS varies depending on the stage at which molecular markers are used and the number and values of genes selected.

The objective of our study is to perform a comparative analysis based on case studies. The analysis evaluates costs, time and IP barriers in the obtainment of a specific breeding objective through different technological alternatives (detailed information on the factors considered in the comparative analysis is reported in Chapter 5 on methodology). The aim of the analysis is to define which technical alternative is the most cost-effective for obtaining the specific breeding objective and to compare additional advantages and limitation of the techniques.

McDougall (2011), in his study about cost and time involved in the discovery, development and authorisation of a new plant variety obtained through the use of biotechnology, describes all stages included in this process, as illustrated in Table 8.1. Stages I, II and III represent the phase of preliminary research, which is usually very variable in terms of time and cost and therefore not considered in the analysis of case studies. Stage IV represents the productions of the new plant varieties, once all the necessary knowledge is ready thanks to the previous phase. Stage IV is thus the subject of this comparative analysis between different technical alternatives. Stage V represents the phase of field trials to multiple the obtained variety and evaluate it under different field conditions. This phase is not included in this comparative analysis since it is the same for all new plant varieties, independently if they are obtained through biotechnology or not. Finally stages VI and VII are undertaken only in the case of varieties included in the scope of GMO legislation; therefore it is taken into account for products of transgenesis and as possible scenario fro products of NPBTs, since they could be eventually classified as GMOs.

The results of the analysis will give an indication about the potential benefit of implementing a new technique for a specific breeding project. Through the help of well-designed case-studies, breeders can make better decisions about choice of the most convenient breeding strategy (Morris *et al.*, 2003).

Table 8.1 Activity stages for discovery and development of a new plant biotechnology derived trait, according to McDougall (2011).

Activity Stage	Definition
I. Early Discovery "Hits"	**Activity**: Preliminary screening and identification of genetic sequences with the potential to deliver the trait of interest. May involve screening genetic libraries, knowledge-based *in silico* genome searches, random activation tagging, gene sequence shuffling, etc. **Output to the next Activity Stage**: genetic sequence "hits".
II. Late Discovery "Leads"	**Input**: genetic sequences **Activity**: Use one or more surrogate model plant system assays (e.g, Arabidopsis, micro-crop), normally with one or two utility promoter cassettes, to evaluate the hits in order to determine which hits may be capable to deliver the trait of interest. This is considered to represent "proof of concept". **Output**: Genetic sequence "leads"
III. Construct Optimization	**Activity**: Lead genetic sequences are combined with different promoter sequences selected for their pattern of constitutive, temporal or tissue-specific expression required to optimize gene expression and gene product accumulation in order to achieve the trait of interest. The target crop is transformed and evaluated under greenhouse and/or field conditions. To evaluate each construct conclusively in plants may be characterized per construct for the trait of interest and no negative agronomic effects. **Output**: Genetic constructs (coding sequence(s) and markers) "leads"
IV. Commercial Event Production & Selection	**Activity**: The Lead genetic constructs are used to product commercial-quality events which are pre-screened using various forms of molecular characterization to eliminate complex or multiple insertions. These events may go through a preliminary evaluation in the greenhouse or nursery as T0 or T1 plants for the trait of interest depending on the complexity of the trait. The numbers may vary depending on the transformation methodology used. **Output**: Commercial-quality events "leads"
V. Introgression, Breeding & Wide-Area Testing	**Activity**: The lead commercial quality events are introgressed into the most elite germplasm to produce sufficient quantities of seed for product-quality hybrids or varieties for evaluation under normal and/or managed field conditions to confirm the trait of interest, to ensure no negative impact of the trait on key performance attributes, yield or grain quality, and to evaluate potential interactions of the event and trait in key product germplasm in multiple environments both alone and with other events. These field evaluations will likely happen over 3-5 years. **Output**: Commercial quality event(s) to regulatory science
VI. Regulatory Science	**Activity**: Conduct all regulatory science studies and data generation in the field, greenhouse, growth chambers and laboratories (internal and external contract research organizations) to fully characterize the event insertion and to confirm the food, feed and environmental safety of products containing the event and representing the trait. The field evaluations may require two seasons to produce the data and prepare the comprehensive data package required for submissions to obtain cultivation and import approvals. **Output**: Regulatory packages to submit for commercial event(s)
VII. Registration & Regulatory Affairs	**Activity**: The staffing resources required to prepare, submit and manage to approval the submissions in 1-2 countries/jurisdictions for cultivation approval and in 5-7 countries/jurisdictions for import approval. Normally 12-15 different agencies. **Output**: Submissions made and approvals obtained for commercial sale and grain production.

8.2 Cisgenesis in wheat

This section is dedicated to a case study in wheat, in which the breeding objective evaluated is the improvement of its bread-making quality. Three alternative plant breeding techniques can be applied to this scope: introgression breeding, transgenesis and the NPBT cisgenesis.

The next sections are meant to describe the interest related to wheat breeding and in particular to bread-making quality, to illustrate the technical possibilities for this objective, and to provide the data from the comparative analysis of costs and time related to each technical alternative.

8.2.1 Bread making quality

Thanks to the unique properties of its flour, wheat is a cereal cultivated worldwide and used to make bread and many other food products such as biscuits, cookies, cakes, breakfast cereal, pasta and noodles. Wheat represents in many countries the most important source of carbohydrates and vegetable proteins and is subject to plant breeding since the nineteenth century.

Wheat genetics is complicated compared to most other cereal species, due to polyploidy (Bancroft *et al.*, 2011). The most common domesticated wheat species are polyploids: as examples durum wheat (*Triticum durum*) is tetraploid, i.e. carries four sets of chromosomes, while the most common bread wheat (*Triticum aestivum*) is hexaploid – six sets of chromosomes. Therefore, many plant breeding techniques are more difficult to apply; especially when the target is the mutation of specific genes that are present in many allelic copies in the polyploidy varieties.

Transgenesis is being applied to wheat since 1992 (Vasil *et al.*, 1992) but with less success compared to other cereals like maize and rice for which transgenic varieties are already cultivated and commercialized in many areas. Due to the strong interest in improving the qualities of this important staple food source, many efforts are being put to improve transformation efficiency and to find technological alternatives for wheat breeding (Shewry and Jones, 2005)

Bread is a widespread food in Europe, European-derived cultures such as the Americas and the Middle East/North Africa. Bread is usually made from dough of wheat flour, which contains gluten proteins that confer elasticity and extensibility to the dough (León *et al.*, 2009). Gluten

proteins consist of monomeric gliadins and polymeric glutenins. Functional properties of bread flour are associated with the content of high molecular weight subunits of wheat glutenin (HMW-GS) (León *et al.*, 2009). It was demonstrated that the effect of HMW-GS on the viscoelastic properties of flour is both quantitative, related to the total amount of HMW subunit proteins expressed, and qualitative, related to differences in the properties of allelic protein subunits (Shewry and Jones, 2005) due to allelic variances. Bread-making quality of wheat can be improved by increasing the proportion of those subunits and, in particular, of some specific alleles associated with the best flour properties.

Bread wheat contains six HMW-GS genes on the long arms of chromosomes 1A, 1B, and 1D (Leon *et al.*, 2010). Allelic variances of these genes result in effects on the structures and properties of the glutenin polymers and hence on bread-making quality of flour (Shewry *et al.*, 2003). Also, D-hordeins from Barley belong to the same family of high molecular weight prolamins as wheat HMW-GS and result to have the same properties in improving bread quality if transferred to wheat (Pistón *et al.*, 2007; Marín *et al.*, 2008).

Different plant breeding techniques can be employed on wheat in order to increase the proportion of high molecular weight prolamins that improve flour quality. The researchers of IAS-CSIC (Institute for Sustainable Agriculture – Consejo Superior de Investigaciones Científicas) in Córdoba, Spain, are working on improvement of cereal products for food and non-food applications since 1992, including wheat and its bread making related characteristics (http://www.ias.csic.es).

The three breeding techniques identified for this case-study (introgression breeding, transgenesis and cisgenesis) are researched by the IAS-CSIC to obtain increased bread making quality in wheat. The techniques are explained in details in the following paragraphs.

8.2.2 Introgression breeding

Introgression breeding (Ballesteros *et al.*, 2003a; Ballesteros *et al.*, 2003b) consists of chromosome substitution for the introduction into wheat of the chromosome 1H from barley that encodes the D-hordeins (gene *HorD*) of interest (Pistón *et al.*, 2007). Chromosome 1H can also be introgressed into wheat from hexaploid tritordeum that derives from crossing *H. chilense* and *Triticum turgidum conv. durum* (2n = 6x = 42, HchHchAABB) (Martin and Sanchez-Mongelaguna, 1982). By using tritordeum, the step of substitution line's preparation is not needed. In case of using barley, substitution lines should be first prepared or ordered. IAS usually makes a request to the Wheat Genetic and Genomic Resources Center (WGGRC) of

Kansas State University – cytogenetic stock (http://www.k-state.edu/wgrc/Germplasm/triticum.html).

As illustrated in Figure 8.1 and in the protocol reported in Box 8.1, introgression breeding technique starts with the cross of the donor line of *tritordeum* and the recipient line of wheat *Triticum aestivum.* The resulting F1 progeny is backcrossed into wheat. From the obtained plants, grains are crushed and protein content is extracted and subject to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in order to identify D-Hordeins, present in arm 1HL, and proteins encoded by genes of arm 1Ds (usually gliadins genes *Gli-1* or *Gli-3*). As consequence we are selecting plant in which a translocation has occurred between chromosomes 1H of *tritordeum* and chromosome 1D of wheat. In this way, the selected final plant will carry only the short arm of chromosome 1H and the lost of information from wheat chromosome 1D is minimised. After 2 more rounds of backcrossing, plants are selected for carrying barley chromosome 1Hs (D-hordeins) and, additionally, for possessing 42 chromosomes, corresponding to 3 complete sets of chromosomes. After self-pollination (generation BC3F2), progeny homozygous for *D-Hor* gene, i.e. possessing the translocation 1Ds-1HL in homozygosity, are selected. Those lines are finally self-pollinated in order to obtain non-segregant lines.

One possibility of shortening the process of selection is the use of the cytogenetic technique FISH (Fluorescent In Situ Hybridization) for selecting the progeny of the first cross and directly identifying the plants carrying the translocation and the proper set of chromosomes. This technique would allow obtaining the desired results in less generation and is more precise to assure that the final product possess the wanted characteristics. However, FISH is very expensive, especially because of the specialised personnel required. Therefore, in IAS they favour the use of the more economic SDS-PAGE technique even if in a longer timeframe. As reported in Box 8.1 the time estimated to carry out the whole process of introgression breeding is 30 months.

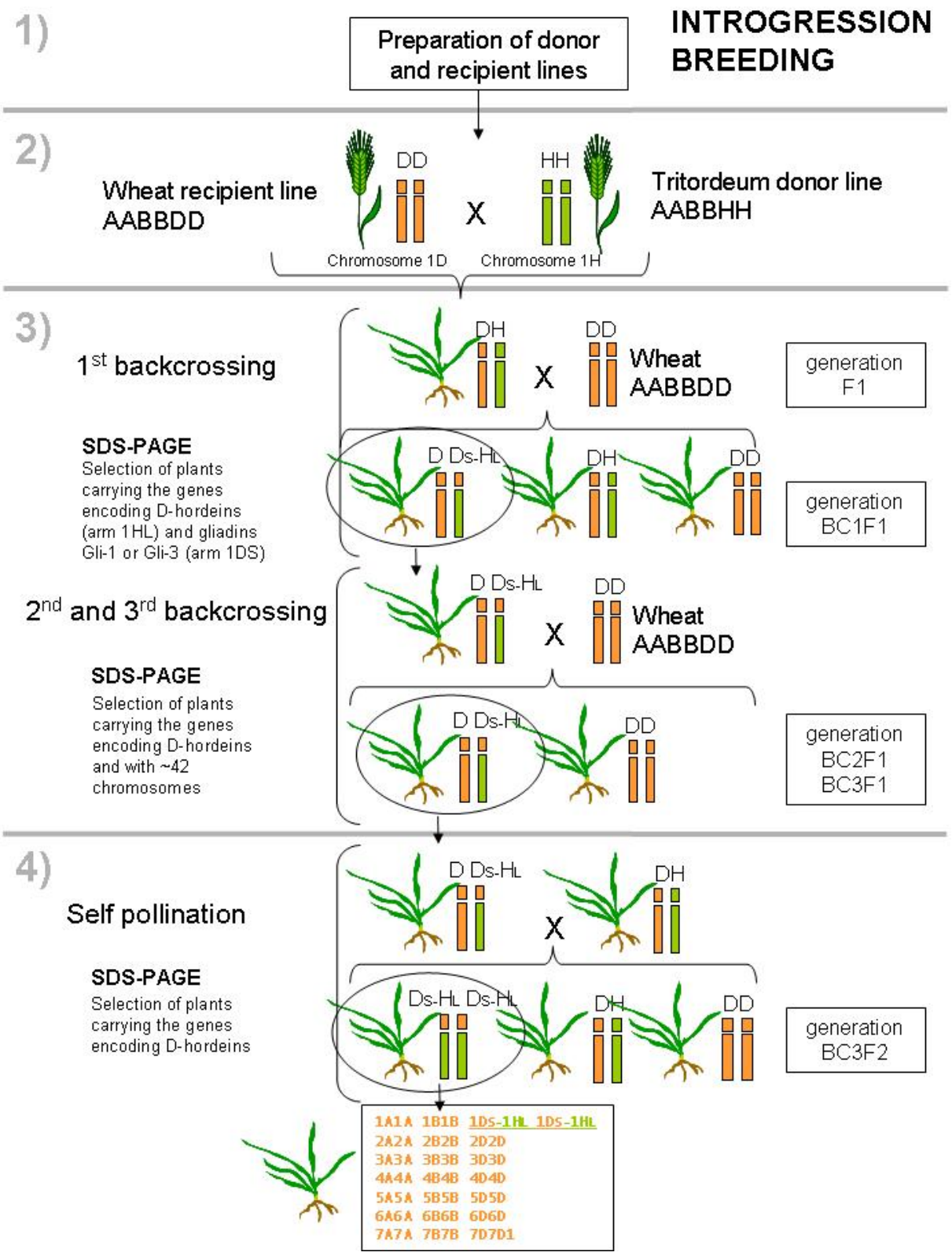

Figure 8.1 Introgression breeding for introduction into wheat of barley chromosome 1H, carrying the genes encoding D-hordeins. The image was developed based on the information supplied by Antonio Martín (IAS-CSIC, Cordoba).

Box 8.1. Introgression breeding protocol

1) PRELIMINARY WORK

- Preparation of donor (barley *Hordeum chilense* or *Tritordeum*...) and recipient (wheat *Triticum aestivum*) lines: Tritordeum line is already available at IAS-CSIC, Barley line can be provided by the Wheat Genetic and Genomic Resources Center (WGGRC) of Kansas State University – cytogenetic stock

  http://www.k-state.edu/wgrc/Germplasm/triticum.html

2) CROSS OF DONOR AND RECIPIENT LINE (Time = 0, duration 6 months)

- First cross of the two lines to obtain the **F_1 generation**

3) BACK-CROSSING AND SELECTION (Time = 6, duration 18 months)

- 1st round of backcrossing with the recipient line to obtain the **BC_1F_1 generation**
- Selection for 1HL (D-hordeins) and 1DS (Gli-1 or Gli-3)

  SDS-PAGE
  - Proteins were extracted from crushed endosperm
  - Gliadins and glutenins extraction
  - SDS- PAGE separation of glutenins and staining
- 2nd and 3rd round of backcrossing with the recipient line (**generations BC_2F_1 and BC_3F_1**)
- SDS-PAGE (see before) for selection of plants with the appropriate N. of chromosomes (e.g. close to 42)
  - probes were labelled by nicktranslation
  - in situ hybridization pattern observed after probing the chromosome preparations
  - Signals were visualized using a fluorescence microscope and images were captured with a CCD camera

4) SELF POLLINATION (Time = 24, duration 6 months)

- 1 round of self pollination of the selected plants (**generation BC_2F_2**)
- SDS-PAGE for selection of plants with the expected genotype.

Total time = 30 months

8.2.3 Transgenesis

In transgenesis, plants are transformed for the insertion of barley genes encoding the D-hordeins and of marker genes to facilitate selection of transgenic plants (Barro *et al.*, 2003; Shewry *et al.*, 2006; Marín *et al.*, 2008; León *et al.*, 2009). A detailed protocol of transgenesis in wheat and a graphic scheme of the technique are presented respectively in Figure 8.2 and Box 8.2. Due to the similarity in the process, both cisgenesis and transgenesis are summarised in the same protocol of Box 8.2.

In transgenesis two plasmids are employed: one contains the transgene encoding D-hordein from barley (Pistón *et al.*, 2007; Marín *et al.*, 2008) and the other plasmid contains the *bar* gene that confers tolerance to the herbicide PPT (phosphinothricin). D-hordeins are under control of their own promoter, while *bar* gene is under control of the maize promoter of the ubiquitin gene. Plasmids are precipitated into gold particles that are bombarded into immature wheat scutella (Barcelo and Lazzeri, 1995). Wheat embryos are subsequently regenerated in a non-selective medium and, after 3 weeks, in medium containing the herbicide PPT, in order to select the transformed plants. Selected plants (T_0 generation) all carry the *bar* gene, but they might carry the transgene or not, since the medium is not selective for D-hordein. DNA extracted from young leaves is subject to PCR to identify also the presence of the transgene D-hordein. Selected plants are self-pollinated to obtain T_1 generation. T_1 seeds are subject to Southern blot in order to identify the proteins D-hordeins. Plants are self-pollinated for further generations until a homozygous progeny of plants carrying the transgenes are identified (usually T_2 or T_3).

One possibility of shortening the technical process is the use of the quantitative Real Time PCR (qRT-PCR) to indentify already in T_1 generation the presence of the transgene and the number of insertion copies, to distinguish between homozygous and not already at this stage. Comparably to FISH in conventional breeding, qRT-PCR is an expensive tool that can be considered in case of urgent need of results, but would not be employed in a routine process. Therefore, IAS favours the use of Southern blot in this technique. The time estimated for the process of transgenesis is 22 months, as reported in Box 8.2.

8.2.4 Cisgenesis

In cisgenesis, plants are transformed for the insertion of wheat genes encoding the HMW glutenin subunits under their own promoter and terminator. Marker genes are co-transformed to facilitate selection of transformed plants (Barro *et al.*, 2003; Shewry *et al.*, 2006; León *et al.*,

2009) and are subsequently selected out in order to obtain only plants with DNA of wheat origin (Barro *et al.*, 2002).

As illustrated in Figure 8.3 and Box 8.2, in cisgenesis two DNA fragments are employed: one containing the sequence of the cisgene encoding the HMW glutenin subunit(s) of interest (*1Dx5, 1Ax1* or *1Dy10*) and the other fragment containing the *bar* gene that confers tolerance to PPT. In cisgenesis, the transformation is performed with DNA fragments instead of plasmids for two main reasons. When using plasmids usually the whole plasmidic DNA is integrated in the plant genome, together with the sequences of interest. Therefore, some extra-sequences will be present in the final product that are not of plant origin and would not be accepted in cisgenic plants according to their definition (see section 1.4.3). The second reason for using fragments in cisgenesis is that plasmids could concatenate and therefore integrate in the same site or in very close sites in plant genome. As explained further on in the cisgenesis protocol, the *bar* gene needs to be segregated out, in order to have only plant genes in the final products. Fragments usually integrate in separate sites and this facilitates their segregation.

HMW glutenin subunits genes are under control of their own promoter, while *bar* gene is under control of the maize ubiquitin promoter. Fragments are precipitated into gold particles that are bombarded into immature wheat scutella (Barcelo and Lazzeri, 1995). Wheat embryos are subsequently regenerated in a non-selecting medium and, after 3 weeks, in medium containing the herbicide PPT, in order to select the transformed plants. Selected plants (T_0 generation) all carry the *bar* gene, but they might carry the cisgene or not, since the medium is not selective for HMW-GS. DNA extracted from young leaves is subject to PCR to identify also the presence of the cisgene HMW-GS. Selected plants are self-pollinated to obtain T_1 generation. T_1 seeds are subject to PCR and Southern blot in order to identify plants expressing the glutenin subunits, but not the marker gene *bar*. This step is essential to obtain plants that only carry DNA from its own species and can be considered as cisgenic. Plants are self-pollinated for further generations until a homozygous progeny of plants carrying the cisgenes are identified (usually T_2 or T_3).

As for transgenesis, also in cisgenesis the use of qRT-PCR can be considered to avoid 1-2 generations (see previous section). Due to similarity in the technical process, for cisgenesis like for transgenesis the time estimate is 22 months, as reported in Box 8.2.

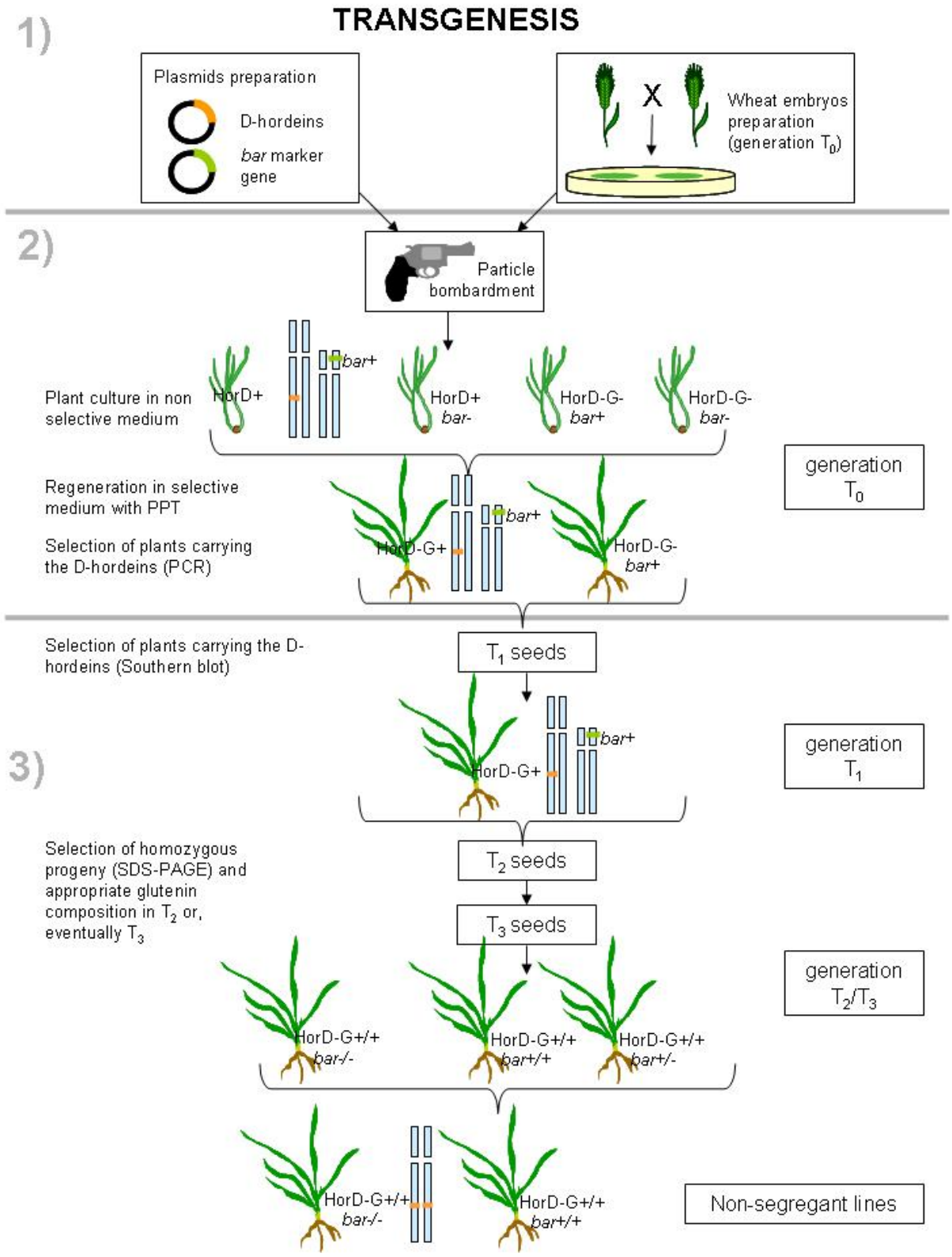

Figure 8.2 Transgenesis for introduction into wheat of barley genes encoding D-hordeins. The image was developed based on the information supplied by Francisco Barro (IAS-CSIC, Cordoba).

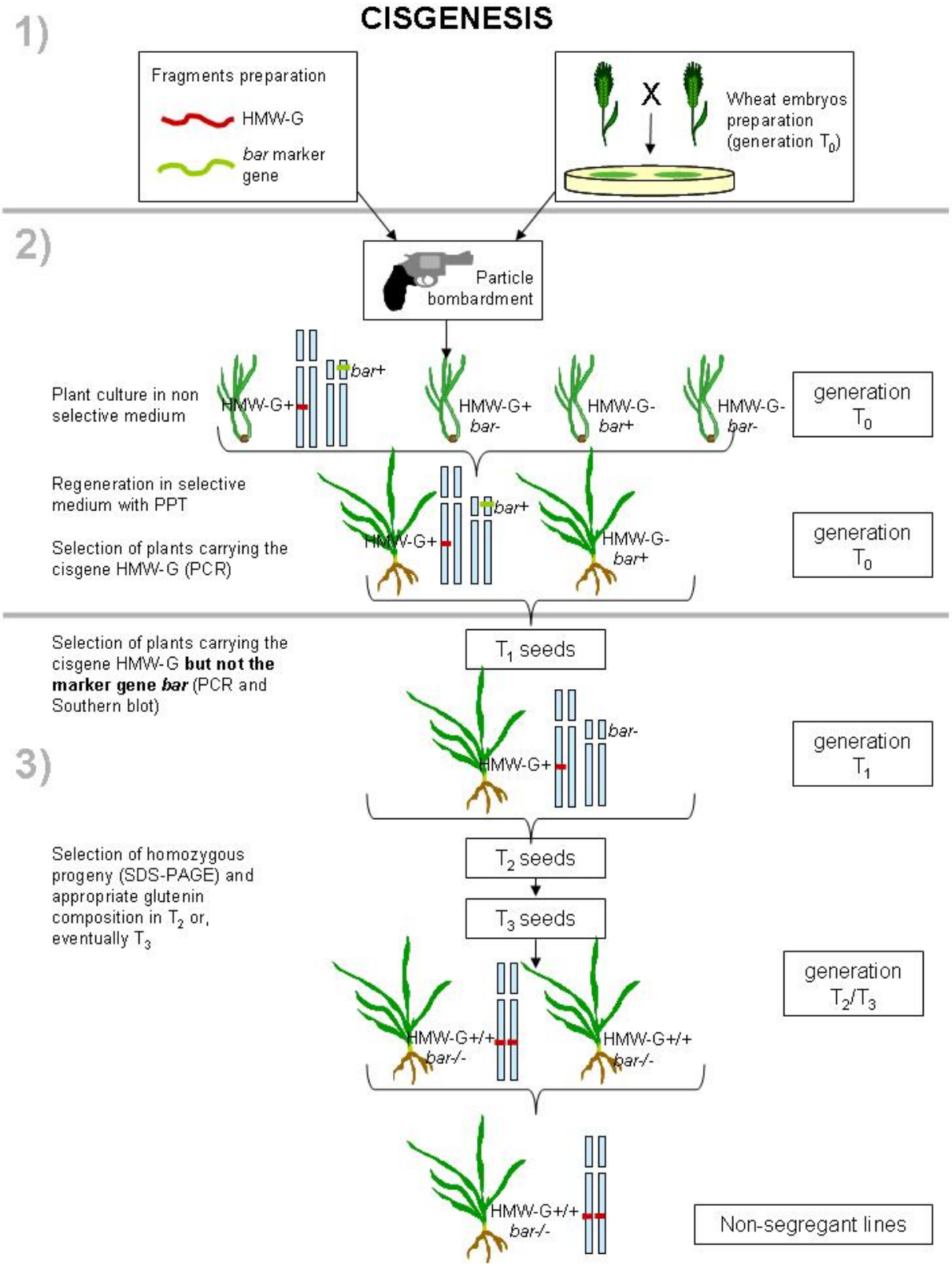

Figure 8.3 Cisgenesis for introduction into wheat of genes encoding HMW-GS. The image was developed based on the information supplied by Francisco Barro (IAS-CSIC, Cordoba).

8.2.5 Comparative analysis

As explained in chapter 5 on methodology, the analysis of costs is based on variable costs, including technical costs, labour, costs of patent licensing and regulatory costs depending if the plant variety is considered as a GMO or not.

The analysis of technical costs has been performed through a spreadsheet-based approach, in which the costs of all steps and materials have been reported separately in order to calculate the final cost of the technique and also to weight all components of the final cost.

Time requirements

As described in the previous paragraph, the estimated duration of the whole process of transgenesis and cisgenesis would be of 22 months, while for introgression breeding it would be a bit longer: 30 months. This time estimations are considered as a realistic option, however, as we already mentioned in the previous paragraph, transformation techniques might become shorter if homozygous lines are identified already in T_1 (1 year shorter) or T_2 (6 months shorter) generation. Also, both for transgenesis/cisgenesis and for introgression breeding, the use of more sophisticated molecular tools (qRT-PCR and FISH, respectively) would allow the breeder to save half year or one year to obtain the final product.

The choice of the starting point for the comparative analysis between the three technical alternatives has clearly a big influence on the time estimation. In fact the starting point was defined as the possession of all knowledge and know-how to develop the plant with the new trait and a fully equipped laboratory. In the case of transgenesis and cisgenesis the starting point taken is when all genetic constructs were known and available. In the case of introgression breeding, the starting point is when the initial substitutions lines are known and available. However, if the starting point were set before the preparation of substitution lines, a few years would be added to the whole process, making the time difference between plant transformation and conventional breeding much bigger. This difference would be reflected also in economic difference, since the potential profit obtainable by the commercialisation of the same wheat variety would be delayed, compared to transformation techniques.

Technical and labour costs

Table 8.1 reports the summary of the technical and labour costs provided by IAS-CSIC for the obtainment of a wheat variety with improved bread-making qualities through the three different technical alternatives: transgenesis and cisgenesis and introgression breeding. The values

reported in Table 8.1 do not include costs related to preliminary phases of material preparation, like fragments or plasmids preparation in the case of transgenesis and cisgenesis and the eventual request of substitution lines in the case of introgression breeding (see section 8.2.2).

According to the data from IAS-CSIC, the technical and labour costs associated to cisgenesis and transgenesis are not significantly different since the two techniques follow a very similar protocol, as described in the previous chapters. For this reason, the two techniques' associated costs are represented in the same column in Table 8.1.

We can first observe that the total technical cost for transgenesis and cisgenesis (€10,149.89) is more than twice the amount required for introgression breeding (€4,426.25). Costs are divided according to the main phases of the technical development. In transgenesis and cisgenesis the first step is embryos transformation, a phase that is not part of the introgression protocol and that includes mainly plasmid isolation (maxiprep), gold particles preparation and GUS test for transformation. According to the data from IAS, the variable cost of this phase corresponds to €1,417.56, which counts for 12% of the total variable costs for obtaining the desired plant variety.

After the transformation step in transgenesis/cisgenesis and after the cross between donor and recipient line in introgression breeding, the next step is in all three cases the selection of plants with the desired genotypic characteristics. In introgression breeding, this involves the analysis of the plant generations through SDS-PAGE. In transgenesis/cisgenesis there is a first phase of growing transformants in regenerative selective medium containing the herbicide PPT to identify the presence of the *bar* gene. This is followed by a molecular selection comparable to the selection phase of introgression breeding through SDS-PAGE, but involving also the use of PCR and Southern blot for the identification of the inserted construct.

In all three techniques, the selection phase accounts for around half of the total cost. According to IAS experts, the total cost of the mediums for the initial selection in transgenesis/cisgenesis is €2,453.83, which includes the cost of the non-selective medium of the first three weeks (€381.12) and of the selective medium for the following 9 weeks (€2,072.71). Many components are included in the latter but the plant hormone zeatin (that promotes callus initiation) covers 50% of the total cost, while PPT counts for 0.04%. The cost of selection with molecular tools like PCR, Southern blot and SDS-PAGE is €4,501.25 for transgenesis/cisgenesis and €2,576.25 for introgression breeding. The difference of €1,925.00 can be attributed to the use of PCR and Southern blot in transgenesis/cisgenesis.

All three techniques are performed in growth chambers until the plant variety is obtained. Therefore, in all three cases we have to consider the costs of plant cultivation for all the generations of the process. This cost is very similar in the three technical alternatives and corresponds to €1,691.65 for transgenesis/cisgenesis and €1,850.00 for introgression breeding. Considering that the order of magnitude of the number of cultivated plants (between 500 and 1000) is similar for the three techniques and that the expensive selective mediums for transgenesis/cisgenesis are calculated separately, it was to be expected that the remaining cultivation costs were similar.

Another parameter to be taken into account in the variable costs is labour. Despite the long duration of the three processes (all around 2 years), the time effectively dedicated to the practical work is much less since there are long time breaks when plants are growing (around 6 months per generation, apart from basic care). Therefore labour costs is not calculated as full-time work during the whole time of the process, but in proportion to the time dedicated to the activity. According to IAS data on labour (reported in Table 8.1), there is not a big difference between labour costs for transgenesis/cisgenesis (€5,990.00 in total) and introgression breeding (€5,340.00 in total).

Table 8.1 Technical and labour costs for obtaining wheat with improved bread-making quality through three technical alternatives: cisgenesis, transgenesis and introgression breeding.

	CISGENESIS TRANSGENESIS costs	**INTROGRESSION BREEDING costs**
TRANSFORMATION		
particle bombardment	€1,417.56	/
PLANT SELECTION		
selection through medium	€2,453.83	/
selection through molecular tools	€4,501.25	€2,576.25
PLANT CULTIVATION	€1,691.65	€1,850.00
EXTRA MATERIAL	€85.60	/
TOTAL TECHNICAL COSTS	**€10,149.89**	**€4,426.25**
LABOUR	€5,990.00	€5,340.00
OVERALL TOTAL COSTS	**€16,139.89**	**€9,766.25**

With the data obtained to this point we can observe that transgenesis/cisgenesis result to be more expensive techniques compare to conventional breeding and this is due in particular to the cost of plant transformation, which is not included in conventional breeding, to the costs of selective mediums, which is in a way a consequence of transformation (the selection of transformants), and to the higher number of molecular tools employed for the selection of plants of interest. Labour is slightly more expensive in transgenesis/cisgenesis (€650 more) than in conventional breeding but, on the other side, transgenesis/cisgenesis can be shorter in terms of time. However, this data has demonstrated to be highly variable in the three techniques, since it depends also on the probability of identifying the desired plant on the first generations and also on the use of more or less expensive tools. Therefore, the main difference so far still relies on technical costs.

Quality of the final product

From a genotypic point of view, the final products of the three techniques described in this section present some differences that may affect their quality. The final product of cisgenesis is in principle the one that presents most genetic similarity with the initial élite variety, since only one extra-gene is inserted into it, conferring the trait of improved bread-making quality. The product of transgenesis, in addition to the extragene for bread-making quality, also carries the marker gene *bar*, that is not present in the initial variety, nor in the cisgenic plant. In principle these two plant products did not loose any genetic material compared to the initial wheat élite variety. On the other hand, products of introgression breeding acquire the new trait of improved bread-making quality together with the whole genetic information contained in the short arm of barley's chromosome 1 (Figure 8.4). This is performed in homozygosis, meaning that the information previously contained in the short arm of wheat's chromosome 1 is lost. Therefore, the final product of conventional breeding in this case might have lost some characteristics of the commercial variety.

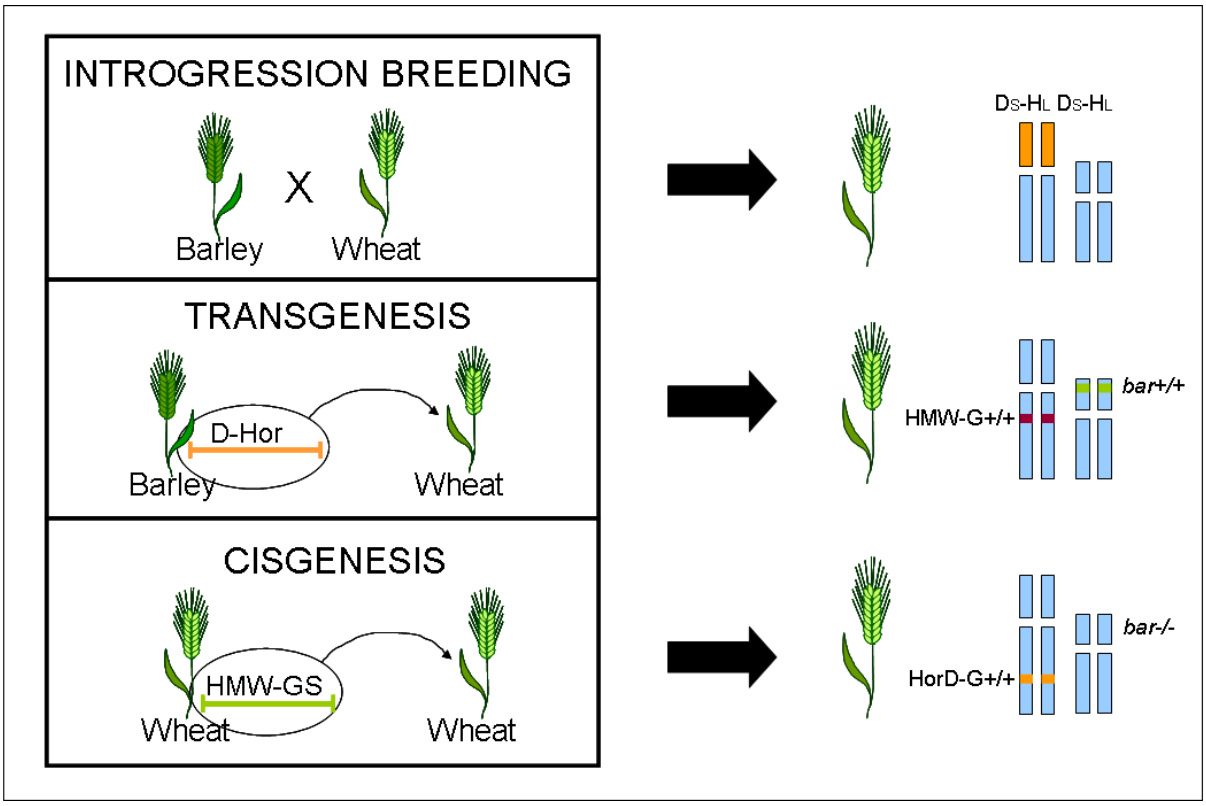

Figure 8.4 Genotypic differences in the final products of the three plant breeding technique.

To the parameters described in the previous paragraphs (time requirements, technical and labour costs) there are two fundamental factors to be taken into account by the breeder that wants to commercialise the variety: patent licensing costs and regulatory requirements.

Patent licensing

In the wheat case-study, it is considered that the final variety is produced for commercialisation. Therefore, the breeder has to pay the licensing revenues of all patented tools employed in the process. According to IAS data, in the case of introgression breeding, no patented tools are employed, therefore, no additional costs should be considered. Conversely, in transgenesis the breeders make use of biolistic transformation technique (covered by several patents held by Du Pont and Powderject Vaccines), and of the marker gene *bar* (patented by Plant Genetic Systems NV and Biogen NV, now owned by Bayer Crop Science) (Mayer *et al.*, 2004) under the control of the maize ubiquitin promoter Ubi-1 (patented by Mycogen Plant Sciences, now owned by Dow Agro Sciences). Inserted genes (HMW-GS and *HorD*) and promoter sequences are not subject of patents, so they are not associated to any licensing fee. The licence for biolistic transformation

has to be accounted also for cisgenesis, while plasmids are not used. Regarding *bar* gene and its promoter, there might be a different agreement between the companies, considering that they would not be present in the final product.

A part from these components, other steps in the whole process might be subject of a valid patent and thus constitute a patent infringement if the final products are brought to the market. A more thorough FTO analysis (see section 3.4.3) would be required to identify all these steps and the relative licenses needed. However, FTO analysis requires a very complex investigation. Section 8.4 in this chapter is meant to give an overview of the essence of this analysis, applied to case study on grafting on GM rootstock.

As illustrated in section 3.4.4, licensing payments might vary according to the specific agreements taken between licensor and licensee and are usually influenced by the market potential of the final products obtained through the licensing (in this case, the improved wheat varieties). A distinction is also made between enabling technologies, which are usually licensed non-exclusively, and trait technologies, which are often licensed exclusively and normally require more expensive fees (see section 3.4.4). In this case, the genes inserted into wheat to improve its bread-making quality (HMW-GS and *HorD*) are not subject of patents, therefore no traits technologies have to be licensed.

As described in section 3.4.4, there are "fixed" licensing costs, but costs established through negotiation between licensor and licensee and determined by the commercial potential of the product. As a rough estimate, initial licensing fees could be between €50,000 and €100,000.

Regulatory costs

As described in chapter 2, GM and non-GM varieties follow a completely different regulatory pathway. Non-GM plant varieties, like the products of introgression breeding, need only to be registered in the EU to the Community Plant Variety Office (EC, 1994). Variety registration usually takes 2-3 years and estimated costs are around €10,000 for variety. GM plant varieties, like the products of transgenesis, additionally need authorisation to be placed into the market, according to Directive 2001/18/EC (EC, 2001). This authorisation process usually takes minimum 2-3 years worldwide and costs until commercialisation are estimated around 10-15 million €. Cisgenic products will follow one or the other scenario according to the regulatory status that will be assigned to them.

Considering both patent licensing and regulatory costs, transgenesis is by far more expensive than introgression breeding. Cost for cisgenesis may vary according to the agreements with

companies owning the patents and depending on the regulatory status that will be established. In case cisgenesis will be excluded from the scope of Directive 2001/18, the relative protocol will still be more expensive than introgression breeding, especially due to patent licensing costs, unless required patents expire in the meantime (see section 8.4 for more information).

8.2.6 Conclusions

According to the data provided by IAS-CSIC, it is possible to obtain three new wheat verieties with improved bread-making qualities through three technical alternatives: transgenesis for the introduction of the gene HorD from barley, cisgenesis for the introduction of the gene HMW-GS from other wheat species and conventional breeding for the introgression into wheat of the long arm of barley chromosome 1H, which carries HorD genes.

From the analysis of the resulting plant varieties we can conclude that the products of transgenesis and cisgenesis possess higher quality since they have not lost any characteristics of the initial genetic background of bread wheat, while the product of introgression breeding substituted completely the genetic material of a chromosome's arm, thereby loosing the wheat genetic information of that arm. Cisgenesis' products possess the same quality characteristics than the transgenic wheat, with the additional aspect of carrying only genetic material of its own gene pool and therefore no marker genes.

In addition to the advantages described in the previous paragraph, transgenesis and cisgenesis offer a technical option that is often quicker compared to conventional breeding and this is especially true if the protocol for introgression breeding would include the preparation of substitution lines. However, the price of obtainment of transgenic and cisgenic varieties is higher than the price of introgression breeding, both in terms of labour costs (12% more expensive in transformation techniques) and technical costs (130% higher in transformation techniques).

In addition to these costs, what makes transformation techniques more expensive than conventional breeding are licensing revenues for patented tools that may require an initial investment between €50,000 and €100,000 for transgenesis and cisgenesis, while they are not accounted for conventional breeding. Additionally, patent royalties might be required during the whole period of commercialisation of the product. All fees can vary according to the specific agreements taken between licensor and licensees (see Section 3.4.4). Nevertheless, the breeding in some cases might be able to find alternative technical solutions to reduce patent infringements and the payment of royalties (see section 8.4.4 for more detailed information).

Another big obstacle that characterises the commercialisation of transgenic products compared to conventional breeding products is the regulatory burden, with costs estimated around €10-15 millions, compared with €10,000 for variety registration costs of conventional products. The time related to the regulatory phase can also vary between the two techniques: varietal registration's process takes usually two-three years. Transgenic product might be approved (if approved) in a similar timeframe but, depending on the quality of the data provided on the application, there might be unforeseeable delays in the process (see section 2.2.4).

Cisgenesis presents the same advantages of transgenesis in terms of quality of the final product and speed in the technical process. As for transgenesis, this is compensated by a higher cost percentage in the technical obtainment, including labour and needed licenses. In the hypothetical case in which cisgenesis were exempted from EU GMO regulation, there would be only the additional investment for variety registration to be calculated for commercialisation. Therefore, in case of exemption, cisgenesis would bring together all the quality technical characteristics that share with transgenesis and an acceptable final price for the breeder. Therefore, considering the balance of all parameters involved, cisgenesis would be the election technique for obtaining wheat with improved bread-making quality.

8.3 Grafting on GM rootstock in citrus

This section is dedicated to a case study in citrus, in which the breeding objective is to obtain a dwarfing rootstock. A tree with reduced size is of interest for the breeder since it usually produces higher yield per hectare and reduces the costs of treatments. Two alternative plant breeding techniques can be employed to this scope: classical breeding by crossing and selection and transgenesis.

The next sections illustrate the importance of grafting in citrus trees and the interest related to dwarfing plants. The techniques for the obtainment of dwarfing citrus rootstocks are described in details and the data from the comparative analysis of costs and time related to each technical alternative are provided.

8.3.1 The importance of grafting in citrus

The rootstock is an essential element of the tree. Its correct choice influences the tree's productivity and even its life. The rootstock affects a large number of aspects, like adaptation to different types of soils (calcareous soil, salinity, etc.), tolerance or sensitivity to certain pathogens (viruses, fungi, etc.) and influence on fruit quality and productivity.

Careful rootstocks' selection is very important since it is to be a permanent part of an orchard and cannot be changed like a cultural practice, a fertilizer program, and irrigation schedule or pest control procedure (Bitters, 1986). The selection of improved fruit varieties has been performed for centuries, but the choice of the best rootstocks to use was not considered as fundamental prior to a hundred years ago, mainly in the last fifty years.

The use of grafting in citrus culture became a common practice in the middle of the 19th century, as a solution against the outbreaks of "foot-rot" (or gummosis) (Bitters, 1986). Sweet orange (*Citrus sinensis*) varieties revealed to be susceptible to the disease, while sour orange (*Citrus aurantium*) trees were resistant and therefore could be successfully used as rootstocks for the sweet varieties. However, since the first half of the 20th century, the use of sour orange rootstocks started to decline, due to a widespread epidemic of tristeza, a disease caused by Citrus Tristeza Virus (CTV) that led to the death of millions of Citrus trees all over the world. This threat has stimulated the use of alternative resistant varieties as rootstock. In Spain, for instance, the most important rootstock currently used is Carrizo citrange, a hybrid of Sweet orange (*Citrus sinensis*) and Trifoliate Orange (*Poncirus trifoliata*) (Forner-Giner *et al.*, 2003).

Today many hybrid citrus rootstocks are produced and selected to be resistant to the most widespread diseases and to possess the most convenient characteristics in terms of adaptation to different types of soils, fruit quality, etc. The IVIA (Instituto Valenciano de Investigaciones Agrarias), in Valencia, Spain, is particularly active in research and development of new citrus varieties and several hybrid rootstocks produced in IVIA are already commercialised (Forner and Forner, 2004).

8.3.2 Dwarfing citrus

Another aspect of the tree that can be conditioned by the rootstock is the final size. Traditionally, farmers considered as best rootstocks the ones that result in vigorous tall trees. However, the strong international competition forces to improve profitability in agriculture and this can be achieved by increasing productivity and quality of fruits and, in parallel, by reducing the cost of

cultivation. With trees of smaller size, cultivation costs are decreased by reducing the cost of pruning, harvesting and of pesticide treatments. Additionally, in many cases trees with reduced foliage have higher production efficiency, because of the higher density of planting per unit area and consequently the improved yield (Forner and Forner, 2004).

Several studies have been conducted on the mechanisms underlying citrus dwarfing phenotype and influence of a dwarfing rootstock to the scion. Hormonal, anatomical and nutritional mechanisms have been postulated (Lliso *et al.*, 2004). Lliso and co-workers proposed that the dwarfing mechanism relies in differences in carbohydrate assimilation in plant tissues. Other research studies (Vidal *et al.*, 2001; Vidal *et al.*, 2003) have been focusing on the role of gibberellins pathway in trees development. Gibberellins (GA) are plant hormones that function as plant growth regulators influencing a range of developmental processes in higher plants (Davies, 1995). In fact, the application of inhibitors of GA biosynthesis is a usual agricultural practice to reduce plant size (Hedden and Hoad, 1994).

Figure 8.5 illustrates the last steps of GA biosynthesis and the enzymes involved, including GA 20-oxidase (Vidal *et al.*, 2001). Research studies demonstrated that the over-expression of the gene encoding GA 20-oxidase (*CcGA20ox1*), leads to a higher production of GA and consequently to an elongated plant phenotype, as demonstrated in *Arabidopsis* (Huang *et al.*, 1998; Coles *et al.*, 1999) tobacco (Vidal *et al.*, 2001), and citrus (Fagoaga *et al.*, 2007). On the contrary, the down-regulation of GA 20-oxidase through gene silencing results in a diminished production of GA and consequently to a dwarfing phenotype, as demonstrated in *Arabidopsis* (Coles *et al.*, 1999) and citrus (Fagoaga *et al.*, 2007).

In IVIA, two different research groups are focused in developing citrus varieties with dwarfing characteristics. The group of Mª Angeles Forner has a long history of citrus hybrid production through conventional breeding of crossing and selection and produced some hybrid varieties of dwarfing rootstocks. The group of Leandro Peña applies molecular biology tools for the introduction of new traits in citrus plants with known phenotypes and developed a methodology of genetic transformation of citrus plants in order to obtain dwarfing rootstocks that can be grafted with non modified scions.

These two techniques are the subject of the case-study and are illustrated in details in the following sections.

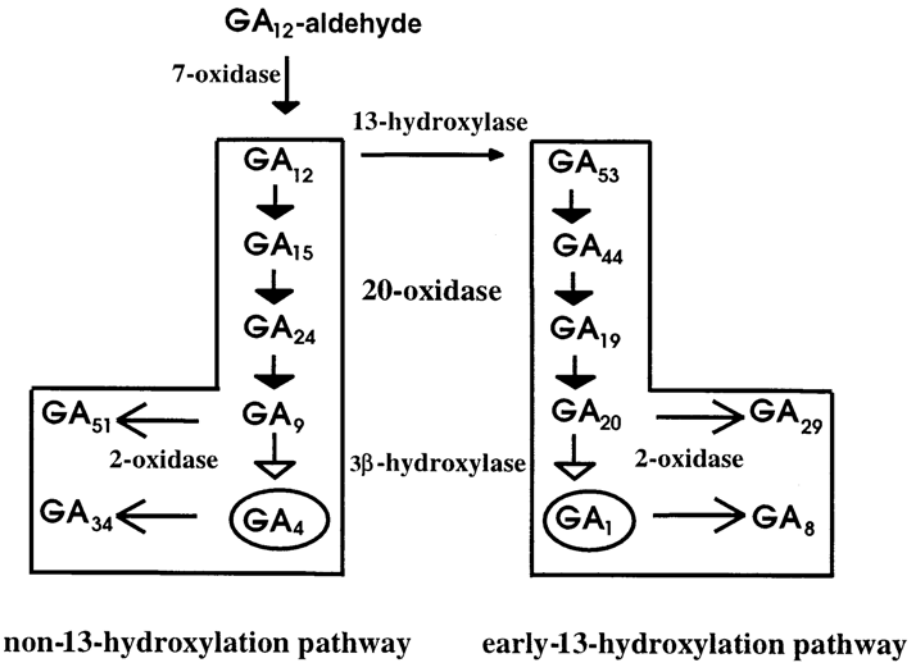

Figure 8.5 Schematic representation of the last steps of gibberellins biosynthesis in plants (Vidal *et al.*, 2001).

8.3.3 Conventional breeding

Conventional breeding in this case study refers to the obtainment of a hybrid new variety of citrus from the crossing of two well known varieties. The initial cross is followed by the selection of progeny with the wanted characteristics of resistance (to certain pests and soil conditions) and, for the described scope, with the dwarfing phenotype.

The protocol for conventional breeding for the obtainment of a dwarfing rootstock variety is schematically illustrated in Figure 8.6 and Box 8.3. The start is the choice of the two parental lines to be crossed. In the chosen example for the case-study, the parental lines are *Poncirus trifoliata* as male parent and *Mandarino King* as female parent. In IVIA, several fields are dedicated to the maintenance of citrus trees to be used as parental lines for the creation of the hybrids of interest. Therefore, the two lines are already available.

The cross between the two lines is performed manually, by collecting the anthers of the male parent and storing the pollen, emasculating the flowers of the female parent to avoid self-pollination and finally depositing the stored pollen to the stigmas of the female parent. Once ripe fruits are obtained, the seeds are collected and cultivated. Hybrids of *Poncirus trifoliata* are

recognised thanks to the trifoliate phenotype. If the male parent were not *Poncirus trifoliata*, then no phenotypic characteristic would be comparable for recognition and therefore MAS technique (see section 1.2.5) would be employed for the recognition of the main characters of the parent, with additional costs. Selected hybrids are multiplied through seeds and sown in the greenhouse. The obtained plants are tested for several parameters, both in the greenhouse and in the field, until they are 10 years old. The characteristics are reported in details in Box 8.3 and include resistance to virus, nematodes and fungi, tolerance to adverse characteristics of the soil, like salinity, drought and chlorosis. Different scions are grafted on the obtained hybrid rootstocks and more parameters are evaluated, like quality and production of fruits, quality of the junction between scion and rootstock, and the final size of the tree to confirm that it is dwarfing.

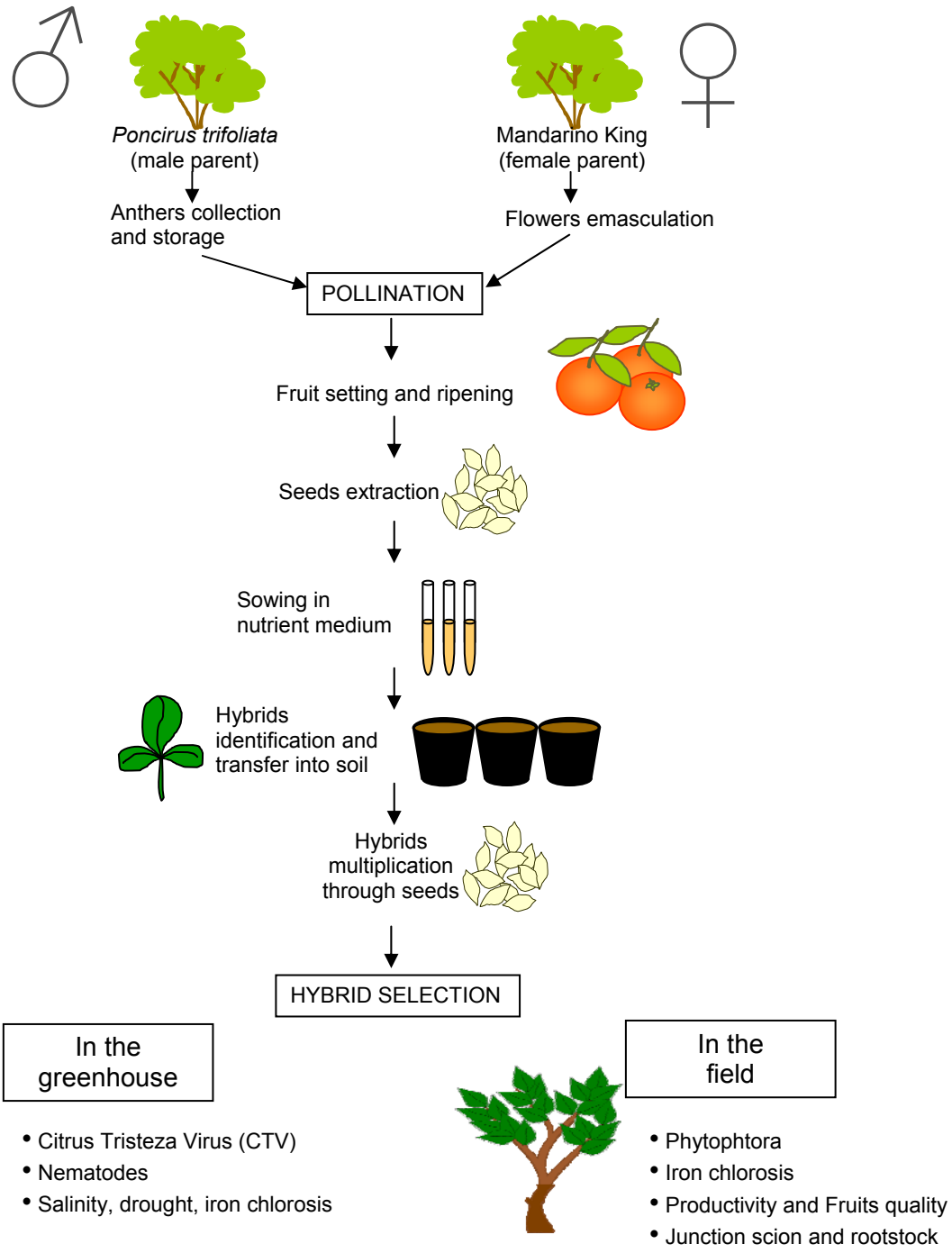

Figure 8.6 Conventional cross breeding for the obtainment of a hybrid citrus rootstock with dwarfing phenotype. The image was developed based on the information supplied by Maria Angeles Forner (IVIA, Valencia).

Box 8.3. Conventional breeding protocol (Forner-Alcaide 517)

1) PRELIMINARY WORK

- Male and female parents trees are chosen and grown (they are already available in the field of IVIA).

2) CROSS OF PARENT LINES TO OBTAIN SEEDS (Time = 0, duration 6 months)

- Anthers are collected from the male parent (*Poncirus trifoliata*), dried in $CaCl_2$ and stored in the fridge at 4ºC or less. Consequently anthers dehiscence occurs and the pollen can be conserved for several weeks.
- Flowers of the female parent (Mandarino King) are emasculated by eliminating the anthers.
- After 24-48h, pollination is carried out by taking the pollen with a small brush and depositing it on the stigmas of the female parent.
- Fruits are let set and ripen. Seeds are collected, peeled, disinfected and sown in test tubes in agar medium with nutrients so that all embryos germinate.

3) HYBRIDS GROWTH AND MULTIPLICATION (Time = 6 months, duration 2.5 years)

- Hybrids are identified (hybrids of *Poncirus trifoliata* can be easily recognised because the trifoliate phenotype is always transmitted to the F1 generation) and transferred into pots with sterilised medium.
- After 6 months, selected hybrids are multiplied through seeds:
    - seeds are sown in the greenhouse, 130 seeds per tray, in a substrate of peat (60%) and sand (40%).

4) HYBRIDS SELECTION (Time = 3 years, duration 10 years)

- When plants are 5 months, they are transplanted to plastic pots, by eliminating the peat from its roots and using silica sand as substrate.
- Hybrids are selected according to the following parameters:
    - In the greenhouse:
        - Resistance/tolerance to Citrus Tristeza Virus (CTV)
        - Tolerance to salinity, drought, iron chlorosis
        - Resistance to Nematodes
    - In the field:
        - Resistance to Phytophtora
        - Iron chlorosis
        - Productivity
        - Fruits quality
        - Junction between scion and rootstock
        - Tree size

Total time = 13 years

8.3.4 Transgenic rootstock

It has been demonstrated that the dwarfing phenotype can be obtained in citrus by silencing the expression of the gene encoding 20 GA oxidase through plant transformation (Fagoaga *et al.*, 2007). This is carried out by introducing to the plant the gene encoding the oxidase in antisense direction, in order to produce post transcriptional gene silencing. The silencing of the endogenous 20 GA oxidase brings to a reduction in the production of GA and consequently in a reduced development of the plant, which is maintained in a dwarfing shape. Figure 8.7 and Box 8.4 provides a detailed illustration of the protocol followed to obtain the dwarfing citrus trees to be used as rootstock. Additional details will be provided in section 8.4 on Freedom to operate.

The first step in the protocol is the preparation of the vector for plant transformation. The gene of interest, *CcGA20ox1*, is isolated from *Carrizo citrange* and cloned in a transformation vector (*pBinJIT*) in antisense orientation, to obtain the silencing effect, and under the control of a strong promoter (*CaMV 35S*) to assure a high level of expression. The vector also contains the marker gene for selection *nptII* for kanamycin resistance and is introduced into *Agrobacterium tumefaciens* through electroporation.

Carrizo citrange is chosen for the transformation, since it possesses very good qualities as rootstock, like good productivity, resistance to waterlogging, frost and Tristeza virus, and is indeed the most widespread rootstock in Spain. The target of plant transformation is to maintain all characteristics of the chosen plant and adding the desired trait of dwarfing size. Buds of *Carrizo citrange* were therefore selected from the bank germplasm in IVIA.

The research group of Leandro Peña has developed a method of transformation of citrus adult material that presents a great advantage compared to older method: plants' juvenile stage is bypassed with the consequent saving of time in the whole breeding process (Cervera *et al.*, 1998). This method has been also patented by the same group (Pena Garcia *et al.*, 1997). The described process consists in first grafting the buds of the plant to be transformed onto a vigorous rootstock, take the internodes of the first sprouting and incubate them with a suspension of *Agrobacteria* carrying the transformation vector. The obtained explants are regenerated in a medium with kanamycin for selection of transformants. In addition, PCR and Southern blot are employed to confirm the presence of the transgene *CcGA20ox1*. The apical portions of the shoots obtained are shoot-tip grafted *in vitro* onto *Troyer citrange* seedlings and again later onto other vigorous rootstocks. PCR and Southern blot are performed on the obtained plants to confirm the stable integration of the *CcGA20ox1* gene.

When the selected transformed plant is about 3 months old, buds are collected and grafted onto vigorous rootstock in the field. However, since the plant carries the transgenes *CcGA20ox1* and *nptII*, it is clearly classified as GMO. Therefore the breeder has to ask for authorisation to the National Competent Authority for its release into the environment for research purposes according to Directive 2001/18 (EC, 2001), as explained in section 2.2. The authorisation process can take up to one year or more but the time cannot be estimated precisely since it depends on the date in which the evaluating committee meets.

Once the transgenic plants are cultivated in the field and are 1 year old, seeds are collected from the fruits and grown in the greenhouse. After 6-8 months, non-GM scions (e.g. from *Clemenules Clementine*) are grafted onto the transgenic rootstocks and after 1-2 months the resulting plants are transplanted into the field. In the field, the phenotypic analysis is performed to confirm that the final plants are dwarfing.

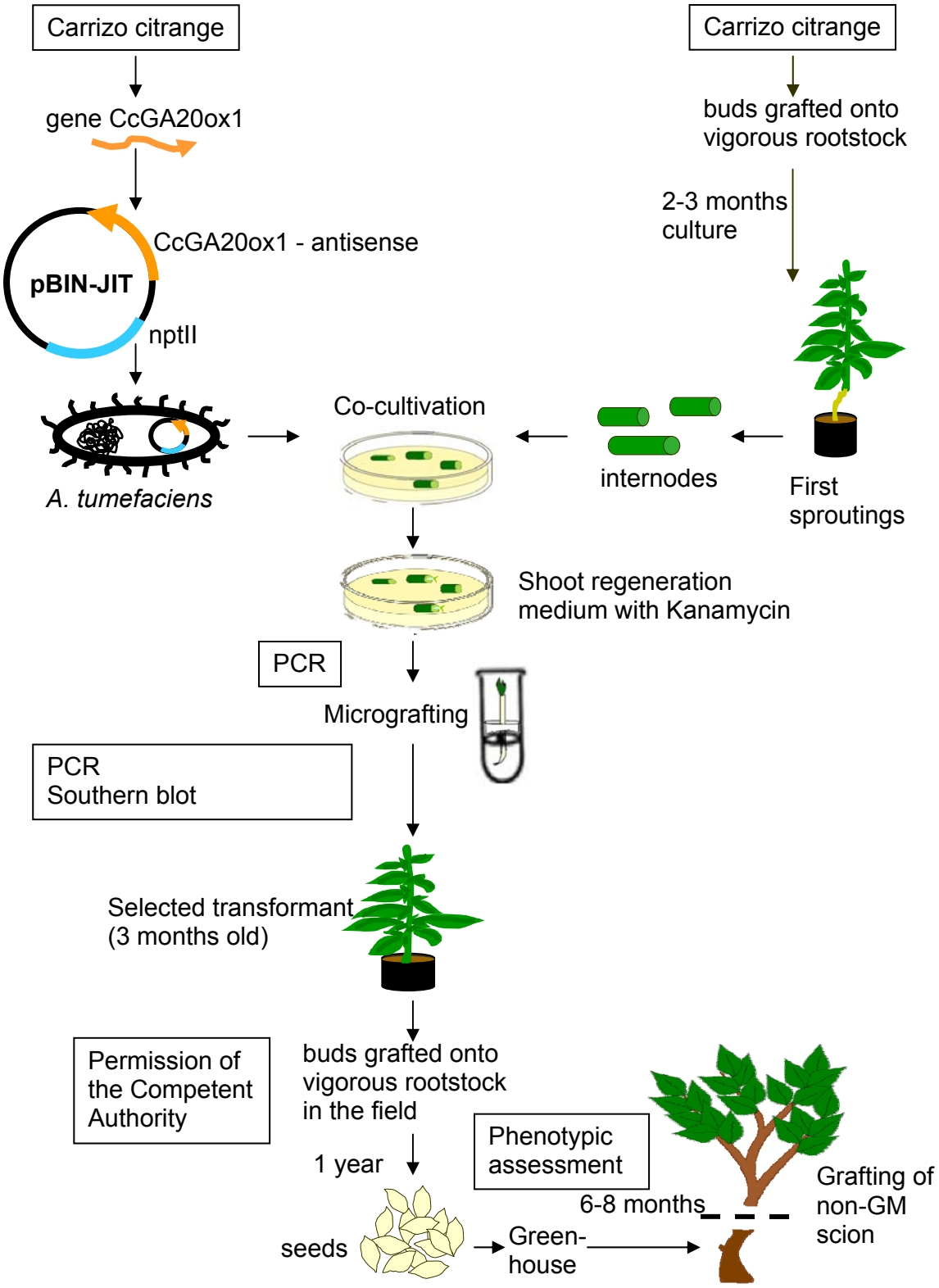

Figure 8.7 Methodology for the obtainment of a transgenic rootstock with dwarfing phenotype. The image was developed based on the information supplied by Leandro Peña (IVIA, Valencia).

Box 8.4. Protocol grafting on a GM rootstock

1) PRELIMINARY WORK

- A cDNA clone of the gene CcGA20ox1 is obtained from *Carrizo citrange*.
- The gene is subcloned in the binary vector pBinJIT (BamHI site) in antisense orientation between the CaMV 35S promoter and the CaMV polyA sequence. The vector pBinJIT also contains the selectable nptII gene (kanamycin resistance) under the *nos* promoter.
- The plasmid is introduced into the disarmed *Agrobacterium tumefaciens* strain EHA105 by electroporation.
- *Agrobacteria* are cultured in selective medium containing kanamycin before being used for transformation.

2) TRANSFORMATION AND TRANSFORMANTS SELECTION (Time = 0, duration 7 months)

- *Carrizo citrange* tree is selected in the germplasm bank. Its buds are grafted onto a vigorous rootstock.
- After 2-3 months the internodes of the first 2-3 sprouting are taken and incubated with the *Agrobacteria* suspension and placed in co-cultivation medium for 3 days (total time = 4 months)
- The explants are transferred to shoot regeneration medium supplemented with kanamycin for selection of transgenic events.
- Genomic DNA is extracted from fresh fully expanded leaves of growing flushes and PCR techniques and Sothern blot are used to detect the presence of CcGA20ox1 gene in the regenerated transgenic plantlets.
- To recover transgenic plants, apical portions of the shoots emerging from the explants are shoot-tip grafted *in vitro* onto *Troyer citrange* seedlings and again later onto vigorous greenhouse-grown rootstock
- PCR and Southern blot are performed to confirm the stable integration of the CcGA20ox1 gene.
- When the selected transformed plant is about 3 months old, buds are collected and grafted onto vigorous rootstock in the field (since the plant is a GMO, the Competent Authority has to release its permission first)

3) GRAFTING OF A NON-GM SCION ONTO THE TRANSFORMED ROOTSTOCK (Time = 10 months, duration 20 months)

- When the resulting plant is 1 year old, fruits are collected to obtain seeds, which are sown in the greenhouse.
- After 6-8 months, non-GM scions (e.g. from *Clemenules Clementine*) are grafted onto the obtained plants and after 1-2 months the resulting plants are transplanted into the field.
- Phenotypic assessment of transgenic plants: Seedlings are also transferred to pots under greenhouse conditions. About 6-7 months later, the following parameters are measured:
    - Number of internodes, branches and leaves
    - Lengths of stems and internodes
    - Leaf area
    - Length of thorns
    - Diameter of main stem and branches

4) OBTAINMENT OF AN ADULT PLANT (Time = 30 months, duration 3 years)

- When the obtained plants are around 2 years old, the first fruits can be collected from the non-GM scion and analyzed for quality parameters.
- From the 3rd year the dwarfing phenotype can be analysed.

Total time = 5.5 years

8.3.5 Comparative analysis

Time requirements

The time estimated for the whole process is much shorter for transgenesis (5.5 years) than for conventional breeding (13 years). The preliminary assumption for time estimation is that through transgenesis only a new gene is added to plant genome to confer a dwarfing phenotype to the plant without altering any other genotypic and phenotypic characteristics. This means that 5.5 years is enough time to confirm the acquisition of the dwarfing trait, having maintained all initial characteristics of Carrizo citrange. This assumption follows the opinion of the developers of this protocol. However, according to many breeders, the modification of a very complex hormonal path as gibberellins synthesis, may have plenty of side-effects on plant development that must be therefore analysed not only in young plants but also in adult plants, since all desired characteristics of the final product have to be anyway confirmed. In this case, the time difference between the two techniques would no be relevant.

A time difference of 7.5 years in the production of a plant variety to be put into the market would represent a clear difference from an economic point of view. In fact, the time delay of conventional breeding can also be converted in cost, since it represents a delay of the benefits of putting the plant into the market.

Technical and labour costs

Table 8.2 reports the costs calculated for the two technical alternatives evaluated for the obtainment of dwarfing citrus rootstocks. At first we can observe that the technical costs calculated for the protocol of conventional breeding are negligible compared to the total cost estimated for trasgenesis that is € 13,215.60.

The most relevant amount in the protocol of transgenesis is the cost associated to plant cultivation during the whole process, which covers the 44% of the total and is attributable in particular to use of growth chambers, compared to conventional breeding.

The second most important component of the total cost is the selection of transgenic plants through molecular tools, which covers 38% of the total. This phase includes the test for histochemical GUS activity, DNA extraction, and PCR and Southern blot techniques for the identification of the transgene inserted.

Plant transformation phase has still an important weight in the whole process, covering 15% of the total cost. The calculated costs takes into account the costs associated to the preparation of

plasmids and fragments, in which expensive restriction enzymes are employed to cut specific DNA sequences, and the purification of the plasmid DNA (through mini and maxi preps). Selective cultivation medium for the identification of antibiotic resistant transformed plants sum a cost of € 378.24, which represents only a 3% of the total.

According to IVIA data (Table 8.2), labour costs for transgenesis (€14,230.00 for the entire process) are more than seven times higher than for conventional breeding (€1,915.00). This big difference is explainable by the fact that transgenesis protocol requires the intervention of more specialised personnel, while conventional protocol only requires the work of a technician.

Quality of the final product

The quality of the final rootstock variety obtained by the protocols described is not the same, since the genotypic background is different. In the case of transgenesis, the genotype of the variety is the same of *Carrizo citrange* with the exception of the new gene inserted. In the case of conventional breeding, two citrus varieties (Mandarino King and *Poncirus trifoliata* in the example of this case-study) are crossed and each plant of the resulting generation takes characteristics of both parents in an unforeseeable way. The selection performed on the offspring allowed IVIA breeders to obtain citrus lines with favourable agronomic characteristics. Table 8.2 shows the comparison between Carrizo citrange (not GM) and other hybrids obtained by cross breeding, including the subject of this case-study, Forner-Alcaide 517.

Table 8.2 Agronomic characteristics of the main commercial citrus hybrid rootstock obtain in IVIA.

		C. CARRIZO	SWINGLE CITRUMELO	M. CLEOPATRA	*C. VOLKAMERIANA*	FORNER-ALCAIDE Nº 5	FORNER-ALCAIDE Nº 13	FORNER-ALCAIDE Nº 517	FORNER-ALCAIDE Nº 418
ASPECTOS VEGETATIVOS Y PRODUCTIVOS	TAMAÑO DE ÁRBOL	●●●●	●●●●	●●●●	●●●●	●●●	●●	●●	●
	PRODUCTIVIDAD	●●●	●●●	●●	●●●●	●●●●	●●●●	●●●●	●●●
	TAMAÑO DE FRUTA	●●●	●●	●●	●●●●	●●●	●●●	●●●	●●●●
	MADURACIÓN	●●●●	●	●●	●●●●	●●●●	●●●●	●●●●	●●●
FISIOPATÍAS	CALIZA	●●	●	●●●●	●●●●	●●●	●●	●●●●	●●
	SALINIDAD	●	●●●	●●●●	●●●	●●●●●	●●●●●	●●●●	●●●
	ENCHARCAMIENTO	●●●	●●●●●	●	●●●	●●●●	●●●●		
	HELADAS	●●●●	●●●●	●●●●	●●	●●●●	●●●●	●●●●	●●●●
PLAGAS Y ENFERMEDADES	TRISTEZA	*Tolerante*	*Tolerante*	*Tolerante*	*Tolerante*	*Resistente*	*Resistente*	*Resistente*	*Tolerante*
	PHYTOPHTHORA	●●●●	●●●●●	●●	●	●●●●	●●●	●●●●	●●
	NEMATODOS	*Sensible*	*Resistente*	*Sensible*	*Sensible*	*Resistente*	*Sensible*	*Resistente*	*Sensible*

Patent licensing

Regarding the estimation of patent licensing costs, according to the experts in conventional breeding, there are no patent infringements in the provided protocol. On the contrary, several patented tools are employed in the protocol for obtaining transgenic dwarfing rootstocks. The analysis of the patented steps involved is presented in the following section on FTO. The same considerations made in the wheat case-study about licensing costs also apply in this case (see section 8.2.5).

Regulatory costs

Regarding regulatory costs, only the variety registration is needed for the product of cross breeding. In the case of the GM rootstock, different scenarios are to be considered, since there is not yet a final decision on the regulatory status of this new technique. If the final plant, constituted by a non GM scion grafted onto the GM rootstock obtained through the described protocol, is considered as a GMO, it needs authorisation to be placed into the market, according to Directive 2001/18/EC (EC, 2001). If the plants obtained through grafting on GM rootstock will be excluded from the scope of GMO legislation, then the same costs of registration would be calculated as in the case of cross breeding products.

For grafting on GM rootstock we can also consider an alternative scenario in which only the environmental risk assessment is required for the cultivation of the GM rootstock, because of the interaction of a GM plant's part with the environment, and no food/feed risk assessment is required for the fruits. The justification for this partial requirement would be that the fruits do not carry any transgenic sequence that may cause harm for human or animal safety when ingested. Additionally, the environmental risk assessment for the rootstock could be reduced considering that no pollen is produced from the rootstock. In this scenario, the costs of the risk assessment would be reduced since the tests strictly associated to food/feed risk assessment would not be required, such as molecular characterisation, toxicological assessment, allergenicity assessment and nutritional assessment (EFSA, 2011). Nevertheless, the requirements for environmental risk assessment are very detailed (EFSA, 2010) and, in case of request for cultivation, represents a very relevant part of the whole assessment. Therefore, the whole price, and especially the time, needed for the deregulation procedure might not be reduced so much by skipping the food/feed assessment.

Table 8.3 Technical and labour costs for obtaining a dwarfing citrus rootstock through two technical alternatives: transgenesis and conventional breeding.

	TRANSGENESIS costs	CROSS BREEDING costs
TRANSFORMATION		
Agrobacterium-mediated	€ 1,684.64	/
PLANT SELECTION		
selection through medium	€ 378.24	/
selection through molecular tools	€4,421.12	/
PLANT CULTIVATION	€ 5,071.60	€100.00
TOTAL TECHNICAL COSTS	**€11,555.60**	**€100.00**
LABOUR	€14,230.00	€1,915.00
OVERALL TOTAL COSTS	**€25,785.60**	**€2,015.00**

8.3.6 Conclusions

There is a great interest among citrus breeders in efficiently obtaining trees with dwarfing characteristics to be employed in intensive agriculture, with a subsequent reduction of costs, in particular related to more dense cultivation, less needs for pruning and less use of chemicals and water. As demonstrated by the scientists working in IVIA, dwarfing citrus rootstocks can be obtained through two different techniques: conventional breeding by crossing two known varieties (*Poncirus trifoliata* and Mandarino King) and transgenesis, in which the gene encoding the GA 20 oxidase is silenced through plant transformation.

According to the data provided on technical and labour costs, costs of transgenesis protocol are clearly higher than conventional breeding, resulting almost 13 times higher, due in particular to the use of the employment of growth chambers for laboratory operations and of expensive molecular tools for plant transformation and transformants selection and for the need of more specialised staff.

On the other hand, transgenesis offers a technical option that is quicker compared to conventional breeding (7.5 years less) if we assume that the genetic modification does not interfere with the rest of phenotype and this time delay represents an economic advantage that might compensate the difference in costs for the protocol.

However, plants obtained through biotechnological tools require inevitably the use of methods and/or materials that are subjects of patents, as it is illustrated in more details in the next section. Therefore, cost for patent licensing are also be included in the comparison. As said in section 3.4.4 those costs are variable and difficult to estimate precisely.

Furthermore, if the plant obtained by grafting a non-GM scion onto the obtained transgenic rootstock will be classified as a GMO, the time difference will be compensated by the time of the authorisation process and high extra-costs would be included in the whole calculation, making the transgenesis option clearly the most expensive.

In conclusion, if grafting on GM rootstock would be excluded from the scope of GMO regulation, the technique would present the advantage of the speed on its conventional counterpart. Technical higher costs would be compensated by the earlier presence of the final products on the market. However, in case of inclusion in the scope of GMO regulation and, probably, also in case of diminished requirements for transgenic rootstock, the conventional method would still be considered as more convenient from an economic point of view. From the point of view of the quality of the final product, two questions remain open: i) if the transgenic product would maintain all the desired characteristics of the non-transformed initial variety and ii) if it would be always possible to obtain the wished new characteristic by cross-breeding.

8.4 Analysis of FTO (Freedom to operate)

FTO analysis is usually performed with the objective of identifying all IP rights (IPRs) needed for the commercialisation of a specific product. These are related to the steps in the obtainment of the product that are protected by patents or other IP rights. Early planning and knowledge about the patent landscape are critical for ensuring that commercial production and subsequent marketing do not infringe the IPRs of others and allow to planning in advance the in-licensing strategy to secure all needed permissions.

This section is meant to illustrate how an analysis of FTO is performed for plant breeding products in which biotechnological tools are employed. The case study of developing a dwarfing citrus rootstock was taken as example for this analysis. This case study was chosen because it represents a real case in which the final variety is close to commercialisation. The dwarfing rootstock obtained quickly by means of plant biotechnology is indeed already driving the attention of the market in South America.

As illustrated in section 3.4.3, FTO analysis requires two steps: product deconstruction and product clearance (Kowalski *et al.*, 2002) and these two phases are presented in the next sections for the process of obtaining dwarfing varieties of citrus rootstocks.

The patent search required in performing an FTO analysis attempts to reveal most patents and patent applications that are relevant for the product in question. However, it must be taken into account that the patent search has inevitably some limitations: i) the expertise of the searcher ii) the time lapse between filing and publication of a patent (non-identified patents might be still not published) iii) geographic coverage might be incomplete since records of certain developing nations are not available on electronic patent databases.

Additionally patent landscape is constantly subject to changes over time. Many patents available in the databases have not been granted and that the lag between submission and grant, if granted at all, can be upwards from two-three years, and in many cases much more than this (Dunwell, 2005). Patent claims may often be modified after objections by patent examiners before grant. Some patent applications can be rejected by the examiners and others can be withdrawn by the applicant or lapse. Patents may also be invalidated after grant. These are the reasons why normally FTO reviews are regularly updated based on changes in the technology used and the changes in patent landscape.

The patent search related to the steps indentified in the production of a dwarfing GM rootstock was not performed with the intention of being 100% exhaustive (as it should be for a FTO analysis performed by professionals) but to identify a high number of potential infringements to give an overview of the topic. The main purpose of the analysis is to illustrate that plant breeding processes involving biotechnologies are complex in term of IP since they inevitably touch many protected inventions or materials.

In general, a thorough FTO analysis prior to product commercialisation should be provided by legally trained personnel who typically assume some liability for their advice. In addition to most of the elements of an FTO review, opinions on validity, enforceability, and infringement issues relating to patents in force are also provided. Also a licensed patent attorney is recommended for a thorough interpretation of claims due to the sometimes complex language and the juridical implications.

8.4.1 Product deconstruction

As described in Section 3.4.3, product deconstruction refers to the definition of the technical content of the product and of all the processes and materials used to obtain the final product. As illustrated in, the entire process for the obtainment of dwarfing citrus rootstock through plant transformation was analysed and deconstructed into its patentable components (Table 8.4). The whole process is divided into four main phases: i) cDNA synthesis (including RNA extraction) for the obtainment of the gene encoding GA 20 oxidase to be cloned in the vector for transformation, ii) the preparation of the construct for transformation, in which the genetic sequences carried by the vector are also indicated as potential patent subject, iii) the phase of plant transformation through *Agrobacterium* and finally iv) the selection of transformed plants through molecular tools. All components will be analysed in more details in the next section on patent analysis.

Normally a FTO analyses before the commercial exploitation of a product requires professional support, due to the complexity of the subject, and in particular of patents language and relationships. Therefore, it is highlighted that the purpose of this chapter is mainly illustrative and perhaps not all patented components have been identified, for each component other patents or dominant patents might be involved and not being reported here or, on the contrary, some reported components might not need to be licensed for the use made. In some cases, the rights over a particular component might have been already expired and this will be also analysed in the next section.

Table 8.4 Product deconstruction for dwarfing citrus rootstocks obtained through transgenesis.

Phase of the process	Components
cDNA synthesis	RNA extraction methods (like proteinase K extraction method, phenol-chloroform extraction method, oligo(dT) cellulose column chromatography, etc.) cDNA synthesis PCR for amplification of the gene CcGA20ox1 (GA20 oxidase)
Transformation construct preparation	Cloning in vector pBluescript SK+ Cloning in vector pBin19-JIT60 Genetic elements included in the vectors: • CcGA20ox1 • CaMV 35S promoter • marker gene nptII • nos promoter
Adult plant material transformation	*Agrobacterium* Transformation of adult material
Transformants selection	PCR Southern blot

8.4.2 Patent review

In this section, for each one of the component previously illustrated in Table 8.4 some relevant patents are listed that could be infringed by the use of the component for commercial purposes.

The following sections will first illustrate the IP issues on specific DNA sequences employed in the process (genes or promoters) and will then focus on the methodological steps of the process.

CaMV 35s promoter

CaMV 35S is a promoter isolated from Cauliflower mosaic virus that is widely used for the constitutive expression of transgenes in plant transformation (see section 1.3.3). In this case, it is used to drive the expression of the gene CcGA20ox1.

CaMV 35 promoter is subject of several patents that address different aspects. Some patents owned by the company Monsanto refer to the use of CaMV 53S as entire sequence promoter controlling a heterologous gene (Table 8.5). It should be observed that these patents also claim the use of other sequences in plant transformation, like the marker gene *nptII* and the promoter *nos*, both also included in the protocol subject of the case study analysed here. Other patents owned by the Rockefeller University claim the DNA sequences of the individual subdomains of the 35S promoter and combinations of them (Table 8.6). Finally, some patents granted to the University of British Columbia claim the use of duplicated CaMV 35S enhancer sequences (Table 8.7). A more detailed review of the claimed use of CaMV 35S promoter in available in Cambia website (Roa-Rodríguez, 2007b).

For each patent also all members of the same patent family have been identified in order to determine the geographic coverage and the relative expiry date. The patents of Monsanto seem to be already expired in European countries, while some applications are still valid in the US, Japan and Brazil (Table 8.5). This means that there would be no patent infringements associated to CaMV 35S promoter when producing the GM variety in the EU, but that the breeder would need a license from Monsanto in case of exporting the plant to the US, Japan or Brazil (unless he waits until these patents also expire). Patents owned by Rockefeller University (Table 8.6) seem to be already expired; no additional family members have been identified. Finally, patents owned by University of British Columbia seem to be still valid in Brazil (Table 8.7). It must be highlighted that this situation may change over time as additional applications may be pending and some may lapse.

Table 8.5 Patents assigned to Monsanto on the use of the entire CaMV 35S promoter.

WO8402913	**Chimeric genes suitable for expression in plant cells** **Assignee: Monsanto Co.** **Priority: 17/01/1983**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
US5034322	05/04/1989	23/07/2008	expired
US5352605	28/10/1993	04/10/2011	expired
US5530196	**02/09/1994**	**25/06/2013**	
US5858742	24/06/1996	24/06/2006	expired
US6174724	**04/05/1995**	**16/01/2018**	
US6255560	**11/01/1999**	**11/01/2019**	
EP0131623	16/01/1984	16/01/2004	expired
AT61406	16/01/1984	16/01/2004	expired
BR1101069	**14/05/1997**	**14/05/2017**	
JP6315381	**28/03/1994**	**28/03/2014**	
JP2645217	**28/03/1994**	**28/03/2014**	
JPS60500796	16/01/1984	16/01/2004	expired
JP7014349	16/01/1984	16/01/2004	expired

Table 8.6 Patents assigned to Rockefeller University on the use of subdomains of the CaMV 35S promoter.

US5097025	**Plant promoters** **Assignee: Rockefeller University (US)** **Priority: 01/12/1989**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
US5097025	01/12/1989	01/08/2009	expired
US5110732	**Selective gene expression in plants** **Assignee: Rockefeller University** **Priority: 14/03/1989**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
US5110732	14/03/1989	05/05/2009	expired

Table 8.7 Patents assigned to University of British Columbia on duplicated CaMV 35S enhancer sequences.

US5164316	**DNA construct for enhancing the efficiency of transcription** **Assignee: University of British Columbia (CA)** **Priority: 17/08/1989**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
US5164316	17/08/1989	17/11/2009	expired
US5196525	08/04/1991	23/03/2010	expired
US5322938	17/11/1992	21/06/2011	expired
US5359142	09/03/1994	25/10/2011	expired
US5424200	11/07/1994	13/06/2012	expired
BR1101045	**14/05/1997**	**14/05/2017**	
CA1325191	12/01/1988	14/12/2010	expired

nptII marker gene

nptII is an antibiotic resistance marker gene that confers resistance to kanamycin, neomycin and geneticin and is commonly used in plant transformation (see section 1.3.3). In the case-study analysed *nptII* is used for the selection of transgenic plants carrying also the gene *CcGA20ox1*.

Like for CaMV 35 promoter, also *nptII* is subject of several patents that address different aspects. The use of *npt* gene as part of a chimeric gene construct for plant transformation is protected by the Monsanto patent WO8402913 (Table 8.4). As illustrated before, this patent is still valid in the US, Japan and Brazil.

Additional patents claiming the use of *nptII* in plant transformation are illustrated in Tables 8.8 and 8.9. The company Japan Tobacco has filed patent applications directed to the use of the *nptII* gene in combination with the antibiotic paromomycin for the selection of transformed rice cells (Table 8.8). This patent is still valid in Europe, Canada and China but seems to be only directed to rice cells. A patent attorney should be consulted to establish if the patent is infringed by the use of the gene in citrus.

The National Research Council of Canada has granted patents directed to dual genetic markers composed of fused genes, which provide the reporter marker gene *gusA* and the antibiotic resistance gene *nptII* (Table 8.9). The patent already expired in most places except the US. A more detailed review of the claimed use of marker genes in available in Cambia website (Roa-Rodríguez and Nottenburg, 2003).

Table 8.8 Patents assigned to Japan Tobacco on the use of the marker gene *nptII* in combination with the antibiotic paromomycin.

WO9905296	**Method for selecting transformed cells** **Assignee: Japan Tobacco Inc** **Priority: 23/07/1997**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
WO9905296	23/07/1998	
EP0927765	**23/07/1998**	**23/07/2018**
AU8356898	23/07/1998	abandoned application
CA2265570	**23/07/1998**	**23/07/2018**
CN1239513	**23/07/1998**	**23/07/2018**

Table 8.9 Patents assigned to the National Research Council of Canada on the use of dual genetic markers in a fused gene: *nptII* and *gusA.*

WO921759	**Bifunctional genetic markers** **Assignee: The National Research Council of Canada** **Priority: 28/03/1991**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
WO9217593	30/03/1992		
EP0583258	30/03/1992	30/03/2012	expired
US5639663	**21/01/1994**	**17/06/2014**	
AU1461192	30/03/1992	30/03/2012	expired
CA2107916	30/03/1992	30/03/2012	expired
DE69226696	30/03/1992	30/03/2012	expired

nos promoter

nos promoter is also very commonly used in plants transformation (see section 1.3.3) and in this case study is used to drive the expression of *nptII*. As for *nptII*, also the use of *nos* promoter is described in the patent WO8402913 (Table 8.4) owned by Monsanto. Additional patents have been identified (Table 8.10), most valid in the US, in which *nos* promoter is mentioned in the claims. Again professionals in patent protection should be consulted to determine if the scope of those patents would be infringed by the use of *nos* promoter in this case-study.

Table 8.10 Patents claiming uses of the *nos* promoter.

WO2011082318	**Methods and compositions for the introduction and regulated expression of genes in plants** **Assignee: Pioneer Hi Bred Int.** **Priority: 30/12/2009**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
WO2011082318	30/12/2010	
US2011167516	**30/12/2010**	**30/12/2030**
WO2011082310	**Methods and compositions for targeted polynucleotide modification** **Assignee: Pioneer Hi Bred Int.** **Priority: 30/12/2009**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
WO2011082310	30/12/2010	
US2011165679	**30/12/2010**	**30/12/2030**
WO2012038717	**Transgenic plants** **Assignee: Cambridge Advanced Tech** **Priority: 22/09/2010**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
WO2012038717	06/09/2011	

GA20 oxidase gene

The gene CcGA20ox, encoding the GA 20 oxidase, is the main subject of the case-study since it is employed to confer a new phenotype to the citrus tree. GA 20 oxidase, as illustrated in section 8.3.2, controls the production of gibberellins hormones. While its over-expression results in an elongated phenotype in the transgenic plants, its silencing results in a dwarfing phenotype. In this case-study, the silencing is obtained by expressing the antisense sequence of the gene for GA 20 oxidase.

Several patents claim the use of GA 20 oxidase gene in plant transformation to modify the gibberellins content, for example assigned to Long Ashton Research Station (UK) (Table 8.11) and to SweTree Genomics AB (SE) (Table 8.12). Both patent families are recent and still valid in all countries in which they were filed. Both patents claim the use of the gene for GA 20 oxidase in sense orientation. In patent WO9428141 it is also specified the use in reverse orientation. Another patent claiming the use of the GA20 oxidase gene in both orientations is WO2011065928, *Processes for accelerating plant growth and increasing cellulose yield*, applied

by SCG Paper Public Company Ltd (TH) and was filed on 24/11/2010 (no family members identified).

More patents can be identified on other aspects of the use of the GA 20 gene for plant transformation and several patents have been filed on the use of genes encoding other oxidases that control the content of gibberellins, like for example patent WO0009722, *Methods for controlling gibberellin levels*, assigned to Monsanto.

Table 8.11 Patents assigned to Long Ashton Research Station on the expression of a gibberellin (GA) 20-oxidase gene or reverse GA 20-oxidase sequence in transgenic plants.

WO9428141	**GA 20-oxidase gene sequences** **Assignee: Long Ashton Research Station (UK)** **Priority: 28/05/1993**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
WO9428141	24/05/1994	
EP0703983	**24/05/1994**	**24/05/2014**
US5939539	**27/11/1995**	**27/11/2015**
US6198021	**21/04/1999**	**21/04/2019**
US6455675	**13/12/2000**	**13/12/2020**
AT291626	**24/05/1994**	**24/05/2014**
DE69434312	**24/05/1994**	**24/05/2014**
DK703983	**24/05/1994**	**24/05/2014**
ES2237752	**24/05/1994**	**24/05/2014**
PT703983	**24/05/1994**	**24/05/2014**
AU6929794	**24/05/1994**	**24/05/2014**
CA2163454	**24/05/1994**	**24/05/2014**
JPH08510381	**24/05/1994**	**24/05/2014**

Table 8.12 Patents assigned to SweTree Genomics AB on the expression of a gibberellin GA 20-oxidase gene in transgenic populus.

WO0166777	**Transgenic trees exhibiting increased growth, biomass production and xylem fibre length, and methods for their production** **Assignee: SweTree Genomics AB (SE)** **Priority: 07/03/2000**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
WO0166777	02/03/2001	
EP1261726	**02/03/2001**	**02/03/2021**
US7141422	**27/01/2003**	**27/01/2023**
US7807878	**30/10/2006**	**30/10/2026**
AT327338	**02/03/2001**	**02/03/2021**
DE60119901	**02/03/2001**	**02/03/2021**
DK1261726	**02/03/2001**	**02/03/2021**
ES2264974	**02/03/2001**	**02/03/2021**
PT1261726	**02/03/2001**	**02/03/2021**
AU4131701	**02/03/2001**	**02/03/2021**
AU2001241317	**02/03/2001**	**02/03/2021**
BR0108987	**02/03/2001**	**02/03/2021**
CA2400986	**02/03/2001**	**02/03/2021**
CN1416468	**02/03/2001**	**02/03/2021**
JP2003525618	**02/03/2001**	**02/03/2021**
NZ521066	**02/03/2001**	**02/03/2021**
ZA200206789	**23/08/2002**	**23/08/2022**

Vectors

The vectors used in the transformation process can also be subject of protected invention. According to the protocol provided, the gene CcGA20ox1 is first subcloned in the vector pBluescript SK+ and subsequently in the vector pBin19-JIT60.

For pBluescript, a patent was identified that could protect the use of this type of vectors: patent US2009042249, *Vectors for Cloning*, assigned to Fermentas, UAB (LT) and still valid in the US and in the EU. More patents could be involved in the protection of pBluscript but the patent analysis should be performed by a professional.

For the pBin19 vector, the following patents owned by Chinese organisations have been identified, in which pBin19 plasmid is clearly used as part of the invention:

- CN102206661, *Fusion gene for three sweet potato viruses and interference carrier thereof*
  Assignee: Henan Academy of Agricultural Sciences (CN)
  Priority: 31/03/2011

- CN101240290, *Plant transgene expression vector containing green fluorescence protein gene and its construction method and application*
  Assignee: Hangzhou Normal University (CN)
  Priority: 29/02/2008

- CN1958802, *Plant gene expression carrier, and application*
  Assignee: Guizhou University (CN)
  Priority: 15/11/2006

- CN1683540, *Plant expression carrier for improving plant cuttage taking root and apical dominance and its use*
  Assignee: private (CN)
  Priority: 11/03/2005

The dominant patent protecting pBin19 plasmid should be identified. It might be cited by those patents but cited patents are not displayable in the patent database used. Again the support of a professional might help.

Nucleic acids extraction

The initial phase of RNA extraction and cDNA synthesis might also be included in the FTO analysis. Some examples of patents related to the extractions of nucleic acids are the following:

- US2004167324, *Method for isolating and purifying nucleic acids*
  Assignee: Hitachi, Ltd.
  Priority: 05/06/2001

- JP2004350693, *Method and apparatus for purifying and isolating nucleic acid*
  Assignee: Hitachi, Ltd.
  Priority: 17/09/2004

- US2010286380, *Pretreatment method for extraction of nucleic acid from biological samples and kits therefor*
  Assignee: Becton Dickinson & Company (US)
  Priority: 06/02/2003

- US5346999, *Method of nucleic acid extraction*
  Assignee: Applied Biosystems (US)
  Priority: 18/01/1985

- WO2005089929, *Modified surfaces as solid supports for nucleic acid purification*
  Assignee: Ambion, Inc. (US)
  Priority: 18/03/2004

And one example of patent about cDNA synthesis is the following:

- WO2004070053, *cDNA amplification for expression profiling*
  Assignee: Amersham Biosciences Corp. (US)
  Priority: 03/02/2003

Also here a professional consultancy would determine which ones exactly are involved in the specific case, if they are still valid or not and in which countries and if they need to be licensed.

Agrobacterium

As described in Section 1.3.1, *Agrobacterium* is nowadays one of the preferred means for plant transformation. The use of *Agrobacterium* is however heavily patented. Patents cover several aspect of the transformation and can differ depending on the tissue transformed, on the type of vectors used, etc. This section reports some examples of fundamental patents on the use of *Agrobacterium* for plant transformation.

There are general patents on the use of basic binary vector molecules and the basic methods for their assembly and use for plant transformation, like the ones illustrated in Table 8.13, owned by the University of Leiden and still valid in the US and in Japan. Other patents protect the use of binary vector molecules modified to improve the efficiency of plant transformation, like the ones listed in Table 8.14. Patent US5149645, owned by the University of Leiden is already expired, while patent WO9102070, owned by Syngenta Mogen B.V. is still valid only in the US. Patents WO9621725, owned by Cornell Research Foundation, Inc., and WO0018939, owned by Pioneer Hi-Bred International, are more recent and still valid in all the countries in which the family members were filed.

Due to the scope of the analysis (citrus transformation), we could confine to patents related to transformation of dicotyledons, and not monocotyledos, like the following example, issued to Washington University and still valid in the US:

- US6051757, *Regeneration of plants containing genetically engineered T-DNA*
  Assignee: University of Washington (US); Competitive Technologies, Inc. (US)
  Priority: 14/01/1983

Table 8.13 Patents assigned to the University of Leiden on basic binary vectors and methods for plant transformation.

US4940838	**Process for the incorporation of foreign DNA into the genome of dicotyledonous plants** **Assignee: University of Leiden** **Priority: 24/02/1983**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
US4940838	23/02/1984	10/07/2007	expired
US5464763	**23/12/1993**	**23/12/2013**	
EP0120516	21/02/1984	21/02/2004	expired
JP6209779	**01/12/1993**	**01/12/2013**	
JP60070080	23/02/1984	23/02/2004	expired
NL8300698	24/02/1983	24/02/2003	expired
AT68829	21/02/1984	21/02/2004	expired

Table 8.14 Patents on modified binary vectors and methods for plant transformation.

US5149645	**Process for introducing foreign DNA into the genome of plants** **Assignee: University of Leiden** **Priority: 04/06/1984**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
US5149645	05/12/1989	22/09/2009	expired
EP0176112	03/06/1985	03/06/2005	expired
NL8401780	04/06/1984	04/06/2004	expired
AT52803	03/06/1985	03/06/2005	expired
JP61052294	03/06/1985	03/06/2005	expired
WO9102070	**Process for the site-directed integration of DNA into the genome of plants** **Assignee: Syngenta Mogen B.V.** **Priority: 26/07/1989**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
WO9102070	26/07/1990		
EP0436007	26/07/1990	26/07/2010	expired
US5501967	**06/07/1993**	**06/07/2013**	
NL8901931	26/07/1989	26/07/2009	expired
AT150792	26/07/1990	26/07/2010	expired
DE69030306	26/07/1990	26/07/2010	expired
DK0436007	26/07/1990	26/07/2010	expired
ES2100173	26/07/1990	26/07/2010	expired
JPH04502860	26/07/1990	26/07/2010	expired
WO9621725	**Binary BAC vector** **Assignee: Cornell Research Foundation, Inc. (US)** **Priority: 13/01/1995**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
WO9621725	11/01/1996		
EP0805851	**11/01/1996**	**11/01/2016**	
US5733744	**13/01/1995**	**31/03/2015**	
US5977439	**22/12/1997**	**22/12/2017**	
AT267871	**11/01/1996**	**11/01/2016**	
DE69632576	**11/01/1996**	**11/01/2016**	
JPH11500306	**11/01/1996**	**11/01/2016**	
WO0018939	**Method of plant transformation** **Assignee: Pioneer Hi-Bred International** **Priority: 01/10/1998**		
Family (INPADOC) members	**Filing date**	**Estimated expiry**	
WO0018939	28/09/1999		
EP1117816	**28/09/1999**	**28/09/2019**	
US6265638	**28/09/1999**	**28/09/2019**	
AT313635	**28/09/1999**	**28/09/2019**	
DE69929073	**28/09/1999**	**28/09/2019**	
AU764100	**28/09/1999**	**28/09/2019**	
AU6164899	**28/09/1999**	**28/09/2019**	
CA2344700	**28/09/1999**	**28/09/2019**	

Additional patents are dedicated to *Agrobacterium*-mediated transformation of specific plants, e.g. citrus plants. The researchers of IVIA, together with INIA (Instituto Nacional de Tecnología Agraria y Alimentaria, based in Madrid - Spain), patented a method of transforming adult citrus tree plants. The method presents the advantage of skipping the juvenile period in the transformation process and therefore saving time in the whole breeding process. It is indeed the method followed in the protocol subject of the case-study and the corresponding patent is reported in Table 8.15.

Three patents are cited by the patent of IVIA and INIA:

- WO9625504, *Genetic modification of plants*
  Assignee: Shell International Research (NL)
  Priority: 17/02/1995

- EP0227264, *Transformation and foreign gene expression with woody species*
  Assignee: Calgene, Inc. (US)
  Priority: 14/11/1985 (expired)

- WO9407356, *Transgenic pomaceous fruit with fire blight resistance*
  Assignee: Cornell Research Foundation, Inc. (US)
  Priority: 30/09/1992

This could mean that these three patents are dominant to the technique protected by IVIA and INIA and therefore have to be licensed to carry out the process described in the patent. However, this should be confirmed by a professional in the field.

A very detailed review of *Agrobacterium* and IP issues is available on Cambia website (Roa-Rodríguez *et al.*, 2003).

Table 8.15 Patents assigned IVIA and INIA on *Agrobacterium* transformation of particular adult woody species.

US6103955	**Procedure for the genetic transformation of adult plants of woody species** **Assignee: INIA & IVIA** **Priority: 05/03/1997**	
Family (INPADOC) members	**Filing date**	**Estimated expiry**
EP0870838	**04/03/1998**	**04/03/2018**
US6103955	**05/03/1998**	**05/03/2018**
DE69835918	**04/03/1998**	**04/03/2018**
ES2151338	**05/03/1997**	**05/03/2017**

PCR

PCR techniques are used in the protocol of the case-study to amplify the cDNA of the CA20 oxidase gene and to identify the transformed plants by detecting the presence of the transgene.

PCR was developed in 1983 by the American Nobel Prize Kary Mullis who patented the inventions with the company Cetus Corporation. The technique is currently employed in a big variety of different applications. Given the high profitability of the invention in the field of molecular biology, many companies subsequently asked for the patent rights to the invention (like the pharmaceutical company Hoffmann-La Roche) and apparently the invention is still subject of a patent battle (Dalton, 2000).

There are several patents on PCR and patent families have usually a very high number of members representing several jurisdiction (sometimes hundreds of patents), due the widespread application of the technique. Therefore, only a selection of PCR patents is presented below, mostly limited to European issued patents on basic uses of the technique.

The following are related to fundamental patents about the PCR process, assigned to Cetus Corporation and Hoffmann-La Roche:

- EP0200362B1, *Process for amplifying, detecting, and/or cloning nucleic acid sequences*
  Assignee: Cetus Corporation (US), Hoffmann-La Roche AG (CH)
  Priority: 28/03/1985 (expired)

- EP0201184B1, *Process for amplifying, detecting, and/or cloning nucleic acid sequences*
  Assignee: Cetus Corporation (US), Hoffmann-La Roche AG (CH)
  Priority: 28/03/1985 (expired)

- EP0502589B1, *Kit for use in amplifying and detecting nucleic acid sequences*
  Assignee: Hoffmann-La Roche AG (CH)
  Priority: 28/03/1985 (expired)

- EP0505012B2, *Oligonucleotides for amplifying nucleic acid sequences and attaching a promoter sequence*
  Assignee: Hoffmann-La Roche AG (CH)
  Priority: 28/03/1985 (expired)

The following are patents about the use of the Taq polymerase, assigned to the Swiss company Hoffmann-La Roche:

- EP0258017B2, *Purified thermostable and process and process for amplifying, detecting, and/or cloning nucleic acid sequences using said enzyme*
  Assignee: Hoffmann-La Roche AG (CH)
  Priority: 22/08/1986 (expired)

- EP0395736B1, *Purified thermostable enzyme*
  Assignee: Hoffmann-La Roche AG (CH)
  Priority: 12/01/1988 (expired)

Also the apparatus needed to perform PCR technique is subject of patents, like the following examples:

- EP0236069B1, *Apparatus and method for performing automated amplification of nucleic acid sequences and assays using heating and cooling steps*
  Assignee: PerkinElmer, Inc. (US)
  Priority: 25/02/1986 (expired)

- EP0776967A3, *Heat exchanger for use in a temperature cycling instrument*
  Assignee: PerkinElmer, Inc. (US)
  Priority: 25/02/1986 (expired)

The patent members issued in Europe seem to be already expired. In this case a professional would be required to check the validity in other countries, depending on the specific interest in commercialising the product. Additionally, more specific aspects of the PCR technique should be considered.

Southern Blot

Southern blot technique is employed in the protocol to identify the presence of the transgene in the transformed plants. It is a widespread technique that was developed in 1973 by Edwin Southern and it is still used after several decades. However, the technique was not patented and therefore could be freely distributed within the scientific community. Some more specific applications of Southern blot technique could be subject of patents, but for the simple use in this protocol it should not be required any licenses.

8.4.3 Product clearance

In a professional FTO analysis, product deconstruction is followed by the phase of product clearance. The person interested in commercialising the final product is requested to assemble and document all information on intellectual property associated to the steps identified: patents, licenses, material transfer agreements etc (Kowalski *et al.*, 2002). This is usually a complex task, especially in public-sector research in which the source of materials used is often untraceable. Often materials like promoters, plasmids or PCR primers are exchanged through several hands vial collegial courtesy.

This phase allows identifying IP infringements that the product developer must remedy to place the product on the market. A professional FTO analysis usually includes suggestions about in-licensing strategy for the products to be commercialised, indicating in particular which institutions should be approached for licensing and at what stage prior to commercialisation.

Product clearance analysis was not performed for this case-study since the objective of this exercise is not to produce a professional FTO analysis but to demonstrate the IP relevance in plant breeding protocol involving biotechnology.

8.4.4 Final considerations

According to the preliminary analysis provided in this section, many elements of the protocol for the obtainment of a dwarfing citrus rootstock through plant transformation may be still subjects of valid patents. However, the need for in-licensing measures in order to guarantee FTO on this protocol seems to be very much related on the specific geographic area in which the final product would be commercialised. In fact, if we consider the patents listed in this section, in most cases they are already expired in Europe and sometimes still valid in the US. This means that marketing the product in the US might result to be more expensive in terms of IP royalties than in Europe.

An option to be considered to secure FTO is the substitution of patented steps with similar method or materials that are not protected by any IPRs or that belong to patents already expired. However, in complex biotechnological innovation it is often not possible to choose these alternative technologies. Finally, other options to obtain the necessary IPRs on the product of interest include negotiations with patent holders, including e.g. cross-licensing, patent pooling and exchange of know-how.

Chapter 9. Overall discussion and conclusions

9.1 Discussion

New plant breeding techniques (NPBTs) have been developed in the last decade as alternatives to conventional and transformation methods. These techniques include techniques of targeted mutagenesis and gene insertion, techniques in which transgenesis is used only as an intermediate breeding step and the final products are free of foreign genes and variants of plant transformation methods, in particular transformation with DNA sequences from cross-compatible plants and grafting techniques in which the upper part does not carry any new DNA sequence.

The field of NPBTs is young and reveals a great potential. According to literature and patents, many institutions are involved in the development of NPBTs: some actors are already known in the market of transgenic products, but also new companies and academies are emerging. The main drivers on the adoption of these NPBTs are their technical potential for introducing desirable traits more precisely in a variety of crops. The main constraints are the regulatory uncertainty about their classification in the scope of the GMO legislation and the limits in freedom to operate that always characterises plant biotechnology.

In the EU, the regulatory uncertainty around these new techniques derives from the fact that they have been developed after the legal definition of GMO was established in the 90s. According to some experts, NPBTs introduce a new continuum between genetic engineering and conventional breeding (2012) since the biotechnological tools employed remind to plant transformation but final products are more similar to conventional breeding products from a genotypic point of view. This intermediate characteristics position NPBTs in the middle between two possible regulatory extremes: the very strict requirements for the risk assessment of GM varieties, with the high related costs, and the absence of risk control for conventionally bred products.

NPBTs seems to be a new phenomenon in plant breeding that drive a lot of attention by researchers and plant breeders, interested in discovering new potential for creation of new plant varieties, but also by regulators and consumers. Similarly as it was for plant transformation in the 80s, also the technological innovation in plant breeding represented by NPBTs has implications in several aspects, not just for the technical potential, but also in the field of regulation, IP and consumer choice. This thesis aimed to address all these aspects of the field of NPBTs and the main results emerged in the analysis are discussed in the following sections.

State of the art

Several institutions are involved in research and industrial development of NPBTs and they belong to different sectors. Multinational companies play a role in this field having produced publications and patents, but most of the activities identified come from small or medium companies and the academic world, in particular from the EU and the US.

What emerges from the analysis of literature, field trials, patent activity and companies' websites is that most NPBTs are technically advanced, proof of concept being obtained already in crops with economic value. Some combinations of crops with traits of interest are concrete products of NPBTs, like e.g. herbicide tolerant oilseed rape and maize obtained through targeted mutagenesis, potato with several traits of interest obtained through cisgenesis and intragenesis and transgenic dwarfing and disease resistant rootstock for grafting of non-GM scions, among others.

All techniques revealed a technical potential and attracted interest by the scientific community. However, the techniques chosen for this analysis revealed to be very heterogeneous in several aspects, such as level of development, obtainable plant-traits combination, products close to commercialisation and main drivers and constraints. Within the group of NPBTs analysed in this thesis, some revealed to be more innovative from the technical point of view. In particular, the techniques of targeted mutagenesis employ new biotechnological tools and are living a boom in terms of publications and patents. Other techniques, like cisgenesis, intragenesis and grafting on GM rootstock, offer less innovative methods, being in practice variants of plant transformation techniques. Finally, the techniques analysed in which transgenesis only represents an intermediate breeding step, RdDM, reverse breeding and early flowering, are novel in scope and methodology but perhaps their industrial potential is still unrevealed. Results per group of techniques are reported and discussed more in detail in the next sections.

Targeted mutagenesis: ODM, ZFN, MGN, TALEN

Targeted mutagenesis techniques represent important alternatives to the existing techniques of mutagenesis applied to plants, which employ chemical or physical mutagens. The added value of targeted mutagenesis techniques is the ability to obtain only one mutation and at the desired sites, and the additional possibility offered by these methods to direct targeted insertion of DNA sequences. The analysis of scientific literature and patent landscape carried out in this thesis in the field of NPBTs revealed that targeted mutagenesis techniques have a particularly dominant role in terms of innovation, potential and interest by industry and academy (Curtin et al., 2012).

The techniques belonging to this group, ODM, ZFN, MGN and TALEN techniques, were first developed for application in human and animal cells as a tool for gene therapy, i.e. the correction of point mutations causing diseases. Indeed most papers and patents on these techniques have been produced about their application in gene therapy. This demonstrates the versatility of targeted mutagenesis techniques whose principles can be applied to several different organisms and this is also on the basis of the high number of scientific publications and patents produced recently on these techniques.

The numbers of scientific publications and patents on targeted mutagenesis techniques in the field of plant breeding are relevant as well and both literature and patent data are growing, revealing their high potential. The first of these techniques to be developed in plants seems to be ODM, with the first patent filed in 1991 and the first papers published in 1999. ODM and ZFN are to date the techniques of targeted mutagenesis subject of the highest number of scientific publications and patents and additionally are described in the papers on NPBTs with the highest impact factor. Furthermore, ODM and ZFN techniques have already been reported to work in crop plants and for trait of agronomic interest, like herbicide tolerance and reduced content of phytates (Shukla et al., 2009). These data suggest that ODM and ZFN techniques are the most advanced in the group of targeted mutagenesis techniques.

On the other side, the use of MGN techniques, largely described in patents and not as much in the scientific literature, have still mainly reported in model plants. TALEN techniques are a very recent discovery that did not yet fully reveal its potential: Very few patents and papers have been produced on TALEN in plants so far, but higher numbers are foreseen for the near future.

US-based institutions seem to have the lead on ODM, both in terms of number of scientific publications and patents. Both academy and industry are present in research and industrial application of ODM in plants. Among the most relevant institutions, the University of Delaware have produced both key scientific publications of high impact factor and key patents, highly cited by the other patents in the field. Private companies, such as Keygene (NL) and Cibus (US) are developing concrete plant products through ODM that are very close to commercialisation, in particular herbicide tolerant crops, among other traits. Herbicide tolerant oilseed rape by Cibus seems to be the product closest to the market.

Similarly to ODM, also scientific literature and patent landscape on ZFN techniques in plants are dominated by US-based institutions and US Universities, in particular Iowa State University, have a leading role in literature. Patent landscape however is clearly dominated by the industry, in particular by Sangamo and Dow companies, being joint assignees in several patents. In ZFN, Utah University appears among the key patents. Also in this case, as for ODM, academic

inventions seem to represent an important basic tool, being cited by subsequent patents from industry. This confirms the observations of Graff et al. (2003), about the public sector playing a fundamental role in several innovative tools in plant breeding.

Several companies seem to be active in the development of commercial plant products obtained through ZFN techniques, in particular Sangamo and Dow, developing a promising herbicide tolerant maize, Danziger and Toolgen. This landscape of different patent owners seems not to coincide with the description of Sangamo monopoly in ZFN application in the medical field (Scott, 2005). Perhaps plant field allows more freedom to different companies. However, considering the very high number of patents filed by Sangamo and Dow it can be foreseen that the other companies will be able to move in a quite narrow field.

Despite the high similarity between MGN and ZFN techniques, the scenario for MGN is quite different. Indeed, EU is the key player in research, with the German Institute IPK (lnstitut fur Pflanzengenetik und Kulturpflanzenforschung) leading in number of scientific publications, and also in patenting activity, with the French company Cellectis dominating in absolute number of patents and possessing highly cited key patents. According to available information, Cellectis and Precision Biosciences are successfully developing plants with useful traits obtained through MGN techniques to be commercialised soon. Compared to ODM and ZFN techniques, which are subjects of many scientific papers and patents, less papers have been produced so far about MGN techniques in plants, maybe due to the fact that the main organisations dealing with the technique (in particular Cellectis) gave priority to patenting than publishing.

For TALEN techniques, EU and US based institutions are comparably present both in scientific literature and patent landscape. However, due to its novelty in the field of plant breeding, it is too soon to identify key actors for TALEN, also considering that no specific products are yet described in research or industrial activities. Nevertheless, TALEN seems to possess a higher potential compared to other targeted mutagenesis techniques, in particular in terms of flexibility, precision, safety and lower technical costs (DeFrancesco, 2011), therefore we can expect a boom of applications in the near future, at least comparable with ODM and ZFN techniques.

Data on possible drivers and constraints for the adoption of NPBTs were extracted from all publications collected as illustrated in the literature sections. The information was integrated with interviews to experts, with the analysis of the practical case-studies and with the survey performed by Lusser et al. (2011). The data retrieved are illustrated in the next paragraphs.

The clearest advantage related to targeted mutagenesis techniques if site-specificity, unlike mutagenesis induced by chemicals or irradiation and transgenesis, which result in random changes in the genome. This is an advantage for the breeder who knows exactly which gene to target and can reach his objective in one step. Site-specificity presents clear advantages from a safety point of view, as discussed in Section 6.3. However, it is not always easy to be technically achieved. In the case of ZFN, MGN and TALEN it requires a complex work of genetic engineering and it is not yet possible to target all sequences. More R&D is required in this field to reach more potential.

Another drawback that needs to be solved is the potential for off-targets as discussed in the section on safety issues. Additionally, the efficiency of the techniques of targeted mutagenesis is not yet very satisfactory. However, estimates for the efficiency of the techniques are variable depending on the crop, the method, the genes involved and marker genes in case they are used. Information in the literature is usually very specific in terms of the plant and genes involved and results are highly variable.

Depending on the specific case, technical constraints emerge in the steps of delivery (of the oligonucleotides or the modified nucleases) and selection. Delivery is particularly complex for ODM, since it involves the preparation of plant protoplasts, and selection is difficult for all the cases in which the trait obtained is not phenotypically selectable, like e.g. herbicide tolerance.

Finally, there are limits in the possibilities offered by targeted mutagenesis if compared to plant transformation, since the genetic variation is restricted to the genes already present in the plants and the traits offered by mutate them.

Variants of plant transformation techniques: Cisgenesis, Intragenesis and Grafting on GM rootstock

In terms of number of publications and patents, this group of techniques follows closely after targeted mutagenesis. As described in the definition provided in section 1.4.3, these techniques share the use of known plant transformation methods (Agrobacterium or biolistic methods) in their protocols. The products obtained through cisgenesis and intragenesis only carry DNA sequences from the host plant species or from cross compatible species, while plants obtained through grafting on GM rootstock only carry transgenic sequences in the rootstock and not in the scion and consequently not in the fruits.

The first scientific paper identified on cisgenesis/intragenesis was produced in 2003. Clearly the transformation of plants with genes from cross-compatible sources or with own genes in

antisense orientation (as accepted in the definition of intragenesis) was experimented already before 2003. However, this date marks the introduction of cisgenesis and intragenesis as concepts of a new group of techniques, with the intention of obtaining products closer to conventionally bred plants. Therefore, from that date also the definition of cisgenesis and intragenesis became more and more detailed and sometimes controversial (Nielsen, 2003; Podevin et al., 2012).

Transformation of plant rootstocks revealed to be a technique already known in 1991, and even before when including all papers produced about research purposes, like e.g. molecular trafficking between rootstock and scion, etc. The real innovation is not the technique itself, but the idea that the plants obtained through this method could be classified in a different way in the light of GMO legislation, in comparison to full transgenic plants.

Compared to the trend of targeted mutagenesis techniques, the techniques described in this group seem not to be subject of a high number of patents, despite the high potential shown by the number of scientific publications. This may be due to the fact that the essential criteria for patentability is that the invention is new and not obvious (see section 3.4.1) and the method employed in these techniques (plant transformation) does not offer real novelty compared to pre-existing patents.

As observed in patent landscape analysis, most patents on NPBTs have mixed claims, which include the process, the tools employed and the final products (see section 7.3). The analysis of the first claim of each patent, as suggested in section 5.3.3, reveals that patents on cisgenesis, intragenesis and grafting on GM rootstock are widely focused on new obtained plant products and new tools employed (as in the example of specific vectors for intragenic transformation, by Simplot), than on the whole plant transformation method. This observation reveals the lower level of innovation for these variants of plant transformation compared to the other new techniques analysed in this thesis.

US and EU-based institutions are again leading in terms of scientific publications and patents produced, but while EU is most active in research for both techniques, US institutions produced more patents. Many patents on these techniques claim the final products obtained, which could be plant species. According to current laws, as explained in chapter 2, plant species are patentable material in the US but not in the EU. This could explain the highest number of US patents for these two techniques.

Compared to the other new techniques analysed, grafting on GM rootstock, cisgenesis and intragenesis have already been used on several crop plants, like potato, apples and watermelon,

and for traits of agronomic interest, like disease resistance and rooting ability, since they rely on existing tools for genetic modification. Main actors for cisgenesis/intragenesis are clearly Wageningen University in the EU and Simplot Company in the US. They both dominate the scene in research activities and in general in retrievable information about products close to commercialisation. Simplot demonstrated to be particularly active in patenting on this technique and its patents are cited by other patents in the group. Cisgenic products of Wageningen University are in particular fungal resistant apples and potatoes, while Simplot is concentrated in producing intragenic potatoes with several traits of interest. Field trials carried out in the EU also revealed additional actors working in apples, potatoes and also barley. In the field of grafting on GM rootstock, several institutions appear in scientific literature, patents and field trials. Many concrete crop plants with potential for future commercialisation appear, but no institution plays the role of main actor and reference for the other players.

What emerges from the case studies on cisgenesis in wheat and grafting on a citrus GM rootstock is that both techniques offer the technical advantages of transgenesis, in terms of speed of the process and maintenance of the quality characteristics of interest in the plant (if we assume that the inserted gene does not interfere with any pre-existing physiological process within the plant cells). However, the employment of biotechnological tools in their protocols, compared to classical breeding, make the whole process more expensive in terms of material employed, specialised labour required and potential patents to be licensed to obtain freedom to operate in the whole process. Considering the very high costs of GM regulation, the eventual classification of these techniques as outside or inside the scope of Directive 2001/18/EC in the EU will have a strong weight on the balance between factors to decide which alternative to choose in a breeding program, if conventional or new plant breeding techniques. More specific drivers and constraints identified for each technique of this group in literature and during experts meetings are illustrated in the following paragraphs.

The technical constraints of cisgenesis, intragenesis and grafting on GM rootstock are associated to the limitations of transformation techniques. As illustrated in Section 1.3, the efficiency of both Agrobacterium-mediated transformation and biolistic methods relies on the specific crop to be transformed but is generally quite low. If T-DNA borders have to be avoided as established in the stricter definition of cisgenesis, only biolistic transformation techniques can be employed. Furthermore, in the case of cisgenesis and intragenesis, the process is limited by the fact that the sequences employed must be chosen from a stricter gene pool available (only cross-compatible sources). For instance, for these techniques, less known and strong promoters are available. Additionally, marker genes must be avoided or segregated/recombined out in the final products, as shown in the case-study on wheat. This implies an additional step compared to transgenesis.

Grafting on GM rootstock combines two breeding techniques with a long history of use: grafting and genetic transformation. Therefore, the technique is well developed, a part from the known limitations of plant transformation as illustrated before. However, knowledge on the movement of molecules from the rootstock to the scion and their influence on gene expression in the scion needs to be further investigated.

Additionally, it is necessary to take into account the possibility of adventitious shoots regenerating from callus (tissue of “bridge” between rootstock and scion) or from rootstock. Fruits originating from these shoots would not present the same genotype as the scion and would carry the transgenic construct like the rootstock.

Techniques resulting in "Negative segregants": Reverse breeding, RdDM, Early flowering

This group of techniques is very heterogeneous in terms of breeding objectives, but very similar form a regulatory point of view when considering a process-based definition of GMO, like the one described in the EU GMO legislation. The common characteristic of the three techniques is that plant transformation is only an intermediate step of the breeding process and no transgenic sequences are present in the final products.

The high number of scientific papers identified on RdDM demonstrates the high interest in the research environment around this technique. However, no clear crop-trait combinations with commercial potential emerge from the scientific literature. RdDM has been applied in a few crop plants but only for the silencing of several marker genes. Additionally, only one patent was identified on this technique, owned by the German Company RLP Agroscience. RdDM confirmed to be innovative and shows a very big potential for research, especially focused on the understanding of the mechanisms behind transcriptional gene silencing, however, the industrial interest seems to be very low, probably because the maintenance of the new traits is not guaranteed through generation and therefore industry does not see the possibility for concrete applications. EU-based research institutions have clearly the lead on this technique, but perhaps only as plant physiological mechanism to be researched in detail, than as a profitable tool in agriculture.

Reverse Breeding confirmed to be a recent and innovative plant breeding technique, owned by the Dutch Company Rijk Zwaan. Papers and patents production is scarce to dateAccording to Heselmans (2011), Rijk Zwaan is using reverse breeding in tomatoes but no clear official information was identified for the moment about potential products that could reach the market.

Early flowering mechanism has been a widely researched topic as demonstrated by the high number of scientific papers identified, already since 1997. However, no clear references about its use for the production of negative segregants with shorter breeding time requirements. According to Waltz (2012) there is a clear commercial interest in early flowering and in particular the Agricultural Research Service of the USDA is using the technique for the acceleration of plum breeding.

Since the techniques belonging to this group include a step of plant transformation and gene integration, the technical constraints are associated to the limitations of transformation techniques, similarly as the ones illustrated for cisgenesis and intragenesis. The additional complexity related to these techniques is the need for a very thorough final selection of the plants that do not carry the transgenic sequence, to assure that the final product is the one claimed in the definition of the techniques.

As previously mentioned, RdDM presents a clear disadvantage related to the quality of the final product. The silencing status after the segregation of the transgene only relies on the epigenetic modification, which is subject to environmental influence and can only be maintained for a certain number of generations. The data about the stability of methylation are still not clear. Additionally, the effect of RdDM is demonstrated to be stronger in silencing transgenes than endogenous genes. For these reasons, the adoption of this technique by the breeding sector is still not clearly predictable.

Reverse breeding presents several complex breeding steps and is not applicable to all kind of crops. In crops with too high ploidy number too many chromosomal combinations would be produced to be screened and crops with seed production problems (like cauliflower) are not suitable for the technical process. The clear advantage of the technique is that its breeding objective – the reproduction of the parental lines of a specific hybrid - cannot be reached with any other conventional technique.

Globally literature search and patent landscape analysis confirmed that the field of NPBTs is very active being most patents and publications produced in the last decade and growing quickly in number. Regarding the amount of patents and scientific publications identified per technique, some techniques revealed to be of potential interest both for research and for industrial applications, while other techniques show a different profile.

Many institutions are involved in the research on NPBTs, mainly from the academic sector and from EU countries. Fewer institutions are active in patenting in the field of NPBTs and most of them, as expected, belong to the private sector. Most of the academic institutions patenting on NPBTs are US Universities.

The reasons behind the paradox of the prevalence of the EU in research activities and of the US in industrial application in the field of NPBTs could be several. As explained in Section 3.2.3, plant variety protection in the US is a form of protection much weaker than plant patents, due to the broad exemptions allowed. Therefore, since plant varieties can be protected in the US through patents, US plant breeding industry could have the tendency of preferring patents instead of variety rights, unlike EU breeders. Another explanation of US prevalence in patenting activities could be a general stronger culture of patenting, compared to the EU, and spread very much in academic institutions. In the EU, a researcher has to decide if to publish or patent his discoveries, since publication is considered as a form of disclosure that hinders the possibility of patenting the same subject. The US system allows protecting a discovery even if it is already described in a scientific publication (by the same researcher).

The geographic coverage of patents identified is very large and spread towards all continents demonstrating that plant breeding industry sees a big potential worldwide for NPBTs.

Multinational companies such as Pioneer, BASF and Bayer appear as authors in some scientific publications and assignees in some patents on NPBTs but in most cases they do not seem to play a key role in the field. It seems that mostly small companies and academic institutes are interested in developing NPBTs, perhaps also encouraged by the intention of circumventing the strict GMO regulation as also observed by Waltz (2012).

Regulatory aspects

Several documents on safety and regulatory issues related to NPBTs emerged from the literature search in the field, in particular on cisgenesis, intragenesis and targeted mutagenesis, being the most advanced techniques. The amount of papers on regulatory issues demonstrates the high attention by scientists to these aspects of new techniques, although with different opinions.

The figures obtained in the case-studies on cisgenesis and grafting on GM rootstock illustrate that regulatory costs related to the classification of final products as GMOs would have a very high weight in the whole balance of the breeding programmes, beyond technical and labour

costs. This high weight is not only due to the costs of the risk assessment analysis to be performed but also to the time uncertainty before (and if) the authorisation is granted.

Therefore, despite the clear technical and economic potential of NPBTs, their application into commercial products is hampered, at least in the EU, until legal clarity is reached about the classification of the products obtained, due to the high costs associated to risk assessment requirements. This distinction is of particular importance for small and medium size enterprises, which play an important role in their development. Classification as GMOs would limit application to traits for high-value crops. Beside high costs, time length uncertainty also represents a limit factor, and in particular for crops marketed only for a few years (Heselmans, 2011).

Consequently, the final decision in terms of classification of NPBTs will clearly determine the direction of the future commercialisation of their products. Companies dealing with products eventually exempted from GMO regulation will clearly be launched in the market. On the other side, products of NPBTs classified as GMOs could be blocked or passed to the hands of bigger companies that can afford the high investments required.

These reasons explain why most publications identified on safety and regulatory aspects of NPBTs report the wish of authors of having the new techniques excluded from the scope of GMO regulation (in the EU or US). The same wish emerged clearly in experts´ meetings and, in particular, during the workshop organised by Lusser et al. (2011) with the participation of experts from academy and small and big enterprises. Only few publications report scientific opinions concerned about safety issues and or expressing the need to regulate certain products of conventional breeding in a similar way as the products of biotechnology.

Many scientists are requesting a science-based approach in the regulation of plant breeding products, claiming that there is a too big gap between the requirements for assessment of products of biotechnology and conventional breeding. Additionally, EU-based scientists state that the EU legislative position must be updated with the advancing scientific evidence; otherwise there is a risk of becoming less competitive than those countries that have more modern regulatory approaches.

Podevin et al. (2012) elaborated a critical analysis of the most important requirements of an appropriate regulatory framework and the challenges that should be faced in the decision about the legal approach towards NPBTs. According to the authors, the regulatory system should be functional over time, provide an equal regulatory oversight for different products that raise similar safety concerns, should stimulate innovation while building consumer trust and provide

an optimal balance between policy objectives and international harmonization. The authors question the capacity of a process-based regulation on GMOs, like the EU system, to face all these challenges, compared to a product-based classification of plant breeding products, like the system in force in Canada.

The legislative approach in the field of agricultural biotechnology varies in different countries. The US legislation is based on case-by-case approach for the evaluation of products of NPBTs and many companies there seem to be active in NPBTs as a possibility of circumventing the strict requirements applied to GMOs (Waltz, 2012). On the other side, EU institutions seem to feel less certain since the classification of NPBTs is not yet established and EU GMO legislation is stricter due to a difference perception of biotech food by the consumers. Therefore, most institutions even possessing patents on NPBTs are waiting final decision before to invest in these new products (Heselmans, 2011).

Regulatory decisions are still under consideration in many countries, and some common elements appear when considering specific NPBTs. However, there is a lack of consensus so far on detailed product definitions and therefore discrepancies in eventual regulatory status for certain NPBTs-products are already pointed out. Podevin et al. (2012) highlights the need for regulatory decisions on NPBTs (and possibly plant breeding techniques in general) to be harmonised at international level. Previous experiences show that asynchronicity in approvals of biotech crops have large consequences for the trade of agricultural products world-wide (Stein and Rodriguez-Cerezo, 2010). A global discussion on the governance of these new techniques seems necessary in the light of previous experiences with current biotech-derived crops and trade disruptions.

What is likely to be expected in the EU as final decision about the legal status of NPBTs is generally that product of plant transformation (cisgenesis, intragenesis and grafting on GM rootstock) may be classified as transgenic plants due to their similarity to transgenic crops in terms of risk assessment requirements. The other new techniques analysed here could be exempted or at least be treated as separate group of techniques, which differ from conventional breeding, but are not detectable as products of biotechnology (Lusser et al., 2011). The availability of detection method is a legal requirement for new GM products to be put into the market, therefore, the impossibility of distinguishing between changes produced by biotechnology tools or conventional breeding will have to be considered in practical terms in the legislation.

IP aspects

What emerged from the analysis of scientific literature and from the comparative analysis through case-studies is that NPBTs are showing great advantages from a technical point of view, in particular in terms of preciseness, quality of the final product and also time-saving aspects, compared to conventional breeding. However, even in the hypothetical case in which regulatory requirements were reduced or eliminated for NPBTs, IP aspects would still be very similar than for transgenic plants, due to the use of biotechnology in the protocols, compared to conventional breeding tools. The strong presence of potential patent infringements in a plant breeding protocol including biotechnology tools was indeed confirmed by the analysis of FTO carried out in the case-study on grafting.

IP access is a general issue for all of crop biotechnology. The reasons lie in the cumulative nature of the genetics and biotechnologies embodied in transgenic varieties. The identification of patents for each one of the NPBTs analysed here is already demonstration of the strong presence of patented elements in plant breeding. A high number of different owners — including companies, individuals, universities and even governments — will have valid IP claims over the technologies and genetic contents that end up being included in it. Additionally, the number of patents in this area is growing at an exponential rate, and therefore IP access could be a deterrent to biotech R&D for years to come (Graff et al., 2004).

Due to the high licensing costs associated, IP requirements constitute therefore a barrier of necessary investments for enterprises with small patent portfolio and in particular for crops with smaller market value like horticultural crop varieties, compared to major raw crops (Graff et al., 2004). To cope with this limitation, an observed tendency for some small companies developing new techniques is to sell the rights to bigger companies, at least for the application in plant breeding, like in the example of Sangamo with ZFN techniques licensed to Dow.

Alternatively, FTO on plant breeding protocol with many patented elements could be secured with different strategies. One option would be to commercialise the product only in geographical areas in which patents were never requested or are already expired. Additionally, the plant breeder could search for alternative steps in the methodology that do not imply valid patented tools. Finally, other options to obtain the necessary IP rights on the product of interest include negotiations with patent holders, including e.g. cross-licensing, patent pooling and exchange of know-how.

An important data that emerged for the analysis of patent landscape in the field of NPBTs is a strong presence of the academic sector in the development of basic tools for the new techniques.

The clearest example is given by key patents on ODM all owned by US-based universities. This phenomenon was already documented in the field of plant transformation techniques by Graff et al. (2003).

Considering the high potential of public sector's discoveries, some initiatives are in place to band together all elements protected by academy in order to reduce their fragmentation and gain FTO in certain fields, like plant breeding. One example is the Public Intellectual Property Resource for Agriculture (PIPRA, www.pipra.org), dealing with US Universities, and CAMBIA (www.cambia.org) based in Australia, with the purpose of developing novel and non-obvious methods for plant modification to circumvent patent infringement.

However, due to the dominance of patents from US based assignees in the field of NPBTs, EU institutions could still be in a weak position regarding freedom to operate, having to license most patents in this field to US-assignees.

Consumer preference

As emerged in the literature analysis, a strong common driver for the adoption of the NPBTs described in this study is the absence of foreign DNA sequences integrated in the final products (with the exception of the case of targeted insertion of transgenes). This characteristic might present advantages from a safety point of view (not clearly demonstrated) but it is surely of great interest from the point of view of consumer acceptance if compared with the reasons behind the refusal of transgenic food.

Consumer acceptance of foods produced with biotechnology means is driven primarily by public perceptions of risks, benefits and safety of these food products. Consumers are often concerned about the naturalness of food products and therefore refuse the introduction into plants of genes from distant species (Rozin et al., 2004). For this reason, they seem to have a more favourable opinion about cisgenic products compared to transgenic (Lusk and Rozan, 2006; Eurobarometer, 2010; Gaskell et al., 2011). However, even cisgenic products are refused by some consumers concerned about the uncontrolled changes that could be caused by the random gene integration. Therefore, it can be expected that techniques for targeted mutagenesis and insertion would receive a favourable acceptance, but no data are yet available.

According to Kuzma and Kokotovich (2011), the proactive engagement of stakeholders and consumers in the regulatory decisions about NPBTs would help to create shared responsibility

and reduce the risk of market failure and mistrust among citizens. The EU is already taken meaningful steps i this direction by commissioning report on specific aspects of NPBTs and in organising public debates (e.g. a public hearing on cisgenesis: http://ecrgroup.eu/?p=4521).

Considering the overall picture given by the analysis of the field of NPBTs and all related aspects, as expressed by Rommens in his work (2010), "R&D efforts in agricultural biotechnology should rely on effective multi-disciplinary teams that interact closely, and communicate openly, with relevant governmental agencies, patent attorneys, industry representatives, and consumer groups".

9.2 Conclusions

The overall conclusions derived from the research conducted in this thesis can be summarized in the points listed below.

1. NPBTs derived from biotechnology have quickly developed in the last decade and the field is still very active, as demonstrated by the analysis of the number of publications and patents in the field, showing a growing trend.

2. Targeted mutagenesis techniques in particular revealed to be very novel, both methodologically and in terms of breeding objectives, and to raise the interest of many actors in the commercial fields, due to their potential applications.

3. Several institutions are involved in the development of NPBTs. The key players are the EU and the US. Among all institutions dealing with NPBTs, many academic organisations and small and medium size enterprises play an important role in terms of research works and patent filing.

4. While the EU have the lead in research activities (number of publications), the US is key-player in patent activity. This difference could be attributed to differences in the patent system for plant breeding, or to a more established and widespread patenting habit in the US, compared to the EU, in particular by public institutions.

5. Some NPBTs are now ready for their application in many crop plants, while others have been tested mainly in model plants. The proof of concept was obtained in several plants for traits of agronomic interest, in particular herbicide tolerance and disease resistance.

6. Some products of NPBTs are already in a phase of development very near to commercialisation, in particular herbicide tolerant oilseed rape, obtained through ODM (Oligonucleotide Directed Mutagenesis), and maize, obtained through ZFN (Zinc Finger Nuclease) techniques, cisgenic and intragenic potatoes with several traits of interest (fungal resistance and improved composition among others) and several fruit trees with disease resistance or dwarfing ability through grafting on GM rootstock.

7. Breeders are interested in NPBTs due to technical and economic advantages. They include in particular their technical potential in terms of preciseness in the introduction of desirable traits and the consequent quality of the final product, time-saving compared

to conventional breeding and, in some documented cases, better consumer acceptance compared to transgenesis.

8. Breeders are very much concerned about the classification of NPBTs in the scope of GMO legislation, especially in the EU, due to the high costs and time uncertainty associated to the approval of regulated products. Therefore, the extent of use of NPBTs will depend on the regulatory status.

9. Discussions on regulatory issues associated to NPBTs are taking place in many countries. Expert groups have been set up to advice on classification of the NPBTs and products in the scope of legislations on crops obtained through biotechnology. The scientific community dealing with NPBTs is making pressure to exempt their products from the legislation.

10. The commercial application of NPBTs is further complicated by the scarce Freedom to operate in the field. Due to the complexity of elements implied in the protocols of plant biotechnology compared to conventional breeding, several patents need to be licensed and licensing costs could hamper investments..

9.3 Conclusiones

Las conclusiones derivadas de la investigación realizada en esta tesis pueden resumirse en los siguientes puntos que se presentan a continuación.

1. El desarrollo de nuevas técnicas de mejora de plantas derivadas de la biotecnología ha aumentado considerablemente en la última década, siendo su potencial de crecimiento todavía muy importante. Este hecho se evidencia en el análisis realizado del número de publicaciones científicas y patentes en ese campo, el cual muestra una tendencia creciente.

2. Cabe destacar que, en particular, las técnicas de mutagénesis dirigidas revelaron ser especialmente novedosas, tanto desde el punto de vista metodológico como de los objetivos derivados de la mejora. Estas técnicas despiertan asimismo un gran interés en el campo comercial debido a sus potenciales aplicaciones.

3. El desarrollo de las nuevas técnicas de mejora de plantas involucra a numerosas instituciones localizadas en su mayoría en la Unión Europea (UE) y Estados Unidos (EEUU). Además del papel que ejercen las grandes instituciones, también destaca un elevado número de organizaciones académicas, y pequeñas y medianas empresas que desempeñan un papel significativo en términos de investigación y registro de patentes.

4. Desde un punto de vista territorial, la UE lidera las técnicas de mejora de plantas en actividades de investigación (número de publicaciones científicas), mientras que los EEUU desempeñan un papel clave en la actividad de patentes. Esta diferencia entre las actividades de investigación y el registro de patentes de mejora de plantas podría atribuirse a diferencias en los sistemas de patentes de la UE y EEUU, o bien al desarrollo de prácticas de patentes más consolidadas y extendidas en los EEUU en comparación con la UE, en particular por parte de las instituciones públicas.

5. Algunas de las nuevas técnicas se encuentran actualmente en una fase de aplicación en cultivos, mientras que otras técnicas siguen en su fase de experimentación en plantas modelo. La prueba de concepto se obtuvo en varias plantas para características de interés agronómico, en particular para aquellas relacionadas con la tolerancia a herbicidas y la resistencia a enfermedades.

6. Algunos productos derivados de las nuevas técnicas están en una fase avanzada, cerca de su comercialización, como es el caso de la colza tolerante a herbicidas, obtenida a través

de la técnica ODM (*Oligonucleotide directed mutagenesis*), el maíz tolerante a herbicidas, obtenido con la técnica de ZFN (*Zinc Finger Nuclease*), las patatas cisgénicas e intragénicas con varias características de interés (como resistencia a hongos y composición mejorada) y varios árboles frutales, con resistencia a enfermedades o enanizantes por injerto sobre un patrón modificado genéticamente.

7. Aquellas instituciones involucradas en las técnicas de mejora de plantas están interesadas en el desarrollo de dichas técnicas debido principalmente a sus ventajas técnicas y económicas. Entre las ventajas destacan en particular, el potencial técnico en términos de precisión en la introducción de características deseables y la consiguiente calidad del producto final, el ahorro de tiempo en el proceso de mejora en comparación con los métodos convencionales y, en algunos casos documentados, la mejor aceptación del consumidor en comparación con los productos transgénicos.

8. Las instituciones involucradas en las técnicas de mejora de plantas están muy preocupadas por la clasificación de estas técnicas en el ámbito de la aplicación de la regulación sobre organismos modificados genéticamente, especialmente en la UE. Esta inquietud se debe principalmente a los altos costes y al tiempo asociado al proceso de autorización. El grado de utilización de las nuevas técnicas dependerá por tanto, de la situación reglamentaria del contexto donde se pretendan implantar los productos derivados de dichas técnicas.

9. Los aspectos derivados de la regulación asociada a las nuevas técnicas de mejora de plantas es un tema de discusión de plena actualidad en muchos países. Se han creado grupos de expertos para asesorar sobre la clasificación de las nuevas técnicas y sus productos en el ámbito de las regulaciones sobre los cultivos obtenidos por medios biotecnológicos. La comunidad científica implicada en el desarrollo de las nuevas técnicas en su mayoría considera que estos productos deberían estar excluidos de la regulación de productos modificados genéticamente.

10. Además de la incertidumbre regulatoria, la aplicación comercial de los productos derivados de las nuevas técnicas se complica aún más por la escasa libertad de operación existente. En concreto, la complejidad de los elementos implicados en los protocolos de la biotecnología vegetal en comparación con los métodos convencionales, requiere que varias patentes deban contar con una licencia, lo cual supone un coste que podría obstaculizar las inversiones en el desarrollo de nuevas técnicas de mejora de plantas.

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Annex I. Literature search results on New Plant Breeding Techniques

Oligonucleotide directed mutagenesis (ODM)

RESEARCH PAPERS

Paper	Country	Plant	Trait
Beetham, P. R., Kipp, P. B., Sawycky, X. L., Arntzen, C. J. & May, G. D. A tool for functional plant genomics: chimeric RNA/DNA oligonucleotides cause in vivo gene-specific mutations. *Proc Natl Acad Sci U S A* **96**, 8774-8778 (1999)	USA	Tobacco	Herbicide tolerance and marker gene
Dong, C., Beetham, P., Vincent, K. & Sharp, P. Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. *Plant Cell Rep* **25**, 457-465 (2006)	AU, US	Wheat	Mutation of marker gene
Gamper, H. B. *et al.* The DNA strand of chimeric RNA/DNA oligonucleotides can direct gene repair/conversion activity in mammalian and plant cell-free extracts. *Nucleic Acids Res* **28**, 4332-4339 (2000)	USA	Canola	Mutation of marker gene
Kmiec, E. B., Johnson, C. & May, G. D. Chloroplast lysates support directed mutagenesis via modified DNA and chimeric RNA/DNA oligonucleotides. *Plant Journal* **27**, 267-274 (2001)	USA	Arabidopsis	Mutation of marker gene
Kochevenko, A. & Willmitzer, L. Chimeric RNA/DNA oligonucleotide-based site-specific modification of the tobacco acetolactate syntase gene. *Plant Physiol* **132**, 174-184 (2003)	DE	Tobacco	Herbicide tolerance
Okuzaki, A. & Toriyama, K. Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. *Plant Cell Rep* **22**, 509-512 (2004)	JP	Rice	Herbicide tolerance
Rice, M. C., May, G. D., Kipp, P. B., Parekh, H. & Kmiec, E. B. Genetic repair of mutations in plant cell-free extracts directed by specific chimeric oligonucleotides. *Plant Physiology* **123**, 427-437 (2000)	USA	Maize, Banana, Tobacco	Mutation of marker gene
Ruiter, R. *et al.* Spontaneous mutation frequency in plants obscures the effect of chimeraplasty. *Plant Mol.Biol.* **53**, 715-729 (2003)	BE	Tobacco, Oilseed rape	Herbicide tolerance
Zhu, T., Mettenburg, K., Peterson, D. J., Tagliani, L. & Baszczynski, C. L. Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nat Biotechnol* **18**, 555-558 (2000)	USA	Maize	Herbicide tolerance
Zhu, T. *et al.* Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. *Proc Natl Acad Sci U S A* **96**, 8768-8773 (1999)	USA	Maize	Herbicide tolerance

REVIEW PAPERS

Paper	Country
Belzile, F. J. Transgenic, transplastomic and other genetically modified plants: a Canadian perspective. *Biochimie* **84**, 1111-1118 (2002)	CA
Breyer, D. *et al.* Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge? *Environ Biosafety Res* **8**, 57-64 (2009)	BE
Britt, A. B. & May, G. D. Re-engineering plant gene targeting. *Trends in Plant Science* **8**, 90-95 (2003)	US
Hohn, B. & Puchta, H. Gene therapy in plants. *Proc Natl Acad Sci U S A* **96**, 8321-8323 (1999)	CH, DE
Iida, S. & Terada, R. Modification of endogenous natural genes by gene targeting in rice and other higher plants. *Plant Mol Biol* **59**, 205-219 (2005)	JP
Kumar, S. & Fladung, M. Controlling transgene integration in plants. *Trends in Plant Science* **6**, 155-159 (2001)	DE

Lokko, Y., Mba, C., Spencer, M., Till, B. & Lagoda, P. Nanotechnology and synthetic biology - Potential in crop improvement. J. Food Agric. Environ. 9, 599-604 (2011).	UN
Oh, T. J. & May, G. D. Oligonucleotide-directed plant gene targeting - Commentary. *Curr. Opin. Biotechnol.* **12**, 169-172 (2001)	US
Puchta, H. Gene replacement by homologous recombination in plants. *Plant Mol Biol* **48**, 173-182 (2002)	DE
Puchta, H. Towards the ideal GMP: Homologous recombination and marker gene excision. *J. Plant Physiol.* **160**, 743-754 (2003)	DE
Reiss, B. in *International Review of Cytology - a Survey of Cell Biology, Vol 228* Vol. 228 *International Review of Cytology-a Survey of Cell Biology* 85-139 (Academic Press Inc, 2003)	DE
Rice, M. C., Czymmek, K. & Kmiec, E. B. The potential of nucleic acid repair in functional genomics. *Nature Biotechnology* **19**, 321-326 (2001)	US
Shewry, P. R. & Jones, H. D. Transgenic wheat: where do we stand after the first 12 years? *Annals of Applied Biology* **147**, 1-14 (2005)	UK
Stewart, C. N., Richards, H. A. & Halfhill, M. D. Transgenic plants and biosafety: Science, misconceptions and public perceptions. *Biotechniques* **29**, 832-+ (2000)	US
Tranel, P. J. & Wright, T. R. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci.* **50**, 700-712 (2002)	US

Zinc finger nuclease (ZFN) techniques (ZFN1,2,3)

RESEARCH PAPERS

Paper	Country	Plant	Trait	ZFN 1,2,3
Cai, C. Q. et al. Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol **69**, 699-709 (2009)	US	Tobacco	Herbicide tolerance	ZFN3
Curtin, S. J. et al. Targeted Mutagenesis of Duplicated Genes in Soybean with Zinc-Finger Nucleases. Plant Physiology 156, 466-473 (2011).	US	Soybean	Mutation of marker gene and endogenous genes	ZFN1
de Pater, S., Neuteboom, L. W., Pinas, J. E., Hooykaas, P. J. & van der Zaal, B. J. ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation. Plant Biotechnol J **7**, 821-835 (2009)	NL	Arabidopsis	Mutation of marker gene	ZFN2
Even-Faitelson, L., Samach, A., Melamed-Bessudo, C., Avivi-Ragolsky, N. & Levy, A. A. Localized egg-cell expression of effector proteins for targeted modification of the Arabidopsis genome. Plant Journal 68, 929-937 (2011).	IL	Arabidopsis	Mutation of marker gene	ZFN1
Lloyd, A., Plaisier, C. L., Carroll, D. & Drews, G. N. Targeted mutagenesis using zinc-finger nucleases in Arabidopsis. Proc Natl Acad Sci U S A **102**, 2232-2237 (2005).	US	Arabidopsis	Mutation of marker gene	ZFN1
Maeder, M. L. et al. Rapid "Open-Source" engineering of customized zinc-finger nucleases for highly efficient gene modification. Molecular Cell **31**, 294-301 (2008).	US	Tobacco	Herbicide tolerance	ZFN1
Marton, I. et al. Nontransgenic Genome Modification in Plant Cells. Plant Physiol. 154, 1079-1087 (2010).	IL, US	Tobacco, Petunia	Mutation of marker gene	ZFN1

Osakabe, K., Osakabe, Y. & Toki, S. Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc. Natl. Acad. Sci. U. S. A. 107, 12034-12039 (2010).	JP	Arabidopsis	Response to abiotic stress	ZFN1
Petolino, J. F. et al. Zinc finger nuclease-mediated transgene deletion. Plant Mol Biol 73, 617-628 (2010).	US	Tobacco	Excision of marker genes	Gene excision
Sander, J. D. et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nat. Methods 8, 67-U94 (2011).	US	Soybean, Arabidopsis	Mutations of endogenous genes	ZFN1
Shukla, V. K. et al. Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature **459**, 437-441 (2009).	US	Maize	Herbicide tolerance	ZFN3
Tovkach, A., Zeevi, V. & Tzfira, T. A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. Plant Journal **57**, 747-757 (2009).	US	Tobacco	Mutation of marker gene	ZFN1
Townsend, J. A. et al. High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature **459**, 442-446 (2009).	US	Tobacco	Herbicide tolerance	ZFN1
Wright, D. A. et al. High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J **44**, 693-705 (2005).	US	Tobacco	Mutation of marker gene	ZFN2
Zeevi, V., Tovkach, A. & Tzfira, T. Increasing cloning possibilities using artificial zinc finger nucleases. Proc. Natl. Acad. Sci. U. S. A. **105**, 12785-12790 (2008).	US	Tobacco	Mutation of marker gene	ZFN1
Zhang, F. et al. High frequency targeted mutagenesis in Arabidopsis thaliana using zinc finger nucleases. Proc Natl Acad Sci U S A 107, 12028-12033 (2010).	US	Arabidopsis	Alcohol resistance and lack of anthocyanins	ZFN1

REVIEW PAPERS

Paper	Country
Alonso, J. M. & Ecker, J. R. Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis. *Nat. Rev. Genet.* **7**, 524-536 (2006)	US
Durai, S. *et al.* Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Res* **33**, 5978-5990 (2005)	US, IN
Jander, G. & Barth, C. Tandem gene arrays: a challenge for functional genomics. *Trends in Plant Science* **12**, 203-210 (2007)	US
Kim, S. & Kim, J. S. Targeted genome engineering via zinc finger nucleases. Plant Biotechnology Reports 5, 9-17 (2011).	KR
Kumar, S., Allen, G. C. & Thompson, W. F. Gene targeting in plants: fingers on the move. *Trends Plant Sci* **11**, 159-161 (2006)	US
Li, J., Hsia, A. P. & Schnable, P. S. Recent advances in plant recombination. *Curr Opin Plant Biol* **10**, 131-135 (2007)	US
Moeller, L. & Wang, K. Engineering with precision: Tools for the new generation of transgenic crops. *Bioscience* **58**, 391-401 (2008)	US
Puchta, H. & Hohn, B. Green light for gene targeting in plants. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 11961-11962 (2005)	DE
Puchta, H. & Hohn, B. Breaking news: plants mutate right on target. Proc Natl Acad Sci U S A 107, 11657-11658 (2010).	DE, CH
Saika, H. & Toki, S. Towards a Highly Efficient Gene Targeting System in Higher Plants. *Jarq - Jpn. Agric. Res. Q.* **43**, 81-85 (2009)	JP

Tzfira, T. & White, C. Towards targeted mutagenesis and gene replacement in plants. *Trends in Biotechnology* **23**, 567-569 (2005)	US, FR
Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 11, 636-646 (2010).	US
Vainstein, A., Marton, I., Zuker, A., Danziger, M. & Tzfira, T. Permanent genome modifications in plant cells by transient viral vectors. Trends in Biotechnology 29, 363-369 (2011).	IL
Weinthal, D., Tovkach, A., Zeevi, V. & Tzfira, T. Genome editing in plant cells by zinc finger nucleases. Trends in Plant Science 15, 308-321 (2010).	US
Wright, D. A. *et al.* Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly. *Nat. Protoc.* **1**, 1637-1652 (2006)	US
Wu, J., Kandavelou, K. & Chandrasegaran, S. Custom-designed zinc finger nucleases: What is next? *Cellular and Molecular Life Sciences* **64**, 2933-2944 (2007)	US

Meganuclease (MGN) techniques (MGN1,2,3)

RESEARCH PAPERS				
Paper	**Country**	**Plant**	**Trait**	**MGN 1,2,3**
Chilton, M. D. & Que, Q. Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. Plant Physiol 133, 956-965 (2003).	US	Tobacco	Mutation of marker gene	MGN 3
D'Halluin, K., Vanderstraeten, C., Stals, E., Cornelissen, M. & Ruiter, R. Homologous recombination: a basis for targeted genome optimization in crop species such as maize. Plant Biotechnol J 6, 93-102 (2008).	BE	Maize	Integration of antibiotic and herbicide resistance genes	MGN 3
Gao, H. R. et al. Heritable targeted mutagenesis in maize using a designed endonuclease. Plant Journal 61, 176-187 (2010).	US	Maize	Mutations of endogenous gene	MGN 1
Gisler, B., Salomon, S. & Puchta, H. The role of double-strand break-induced allelic homologous recombination in somatic plant cells. Plant Journal 32, 277-284 (2002).	DE	Tobacco	Mutation of marker gene	MGN 1
Kirik, A., Salomon, S. & Puchta, H. Species-specific double-strand break repair and genome evolution in plants. EMBO J 19, 5562-5566 (2000).	DE	Arabidopsis and Tobacco	Mutation of marker gene	MGN 1
Kwon, T., Huq, E. & Herrin, D. L. Microhomology-mediated and nonhomologous repair of a double-strand break in the chloroplast genome of Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 107, 13954-13959 (2010).	US	Arabidopsis	Mutations of endogenous gene	MGN 1
Salomon, S. & Puchta, H. Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. Embo Journal 17, 6086-6095 (1998).	DE	Tobacco	Mutation of marker gene	MGN 1
Tzfira, T., Frankman, L. R., Vaidya, M. & Citovsky, V. Site-specific integration of Agrobacterium tumefaciens T-DNA via double-stranded intermediates. Plant Physiology 133, 1011-1023 (2003).	US	Tobacco	Mutation of marker gene	MGN 3

Wehrkamp-Richter, S. et al. Characterisation of a new reporter system allowing high throughput in planta screening for recombination events before and after controlled DNA double strand break induction. Plant Physiology and Biochemistry 47, 248-255 (2009).	FR	Arabidopsis	Mutation of marker gene	MGN 1
Yang, M. Z. et al. Targeted mutagenesis in the progeny of maize transgenic plants. Plant Mol.Biol. 70, 669-679 (2009)	US	Maize	Mutation of marker gene	MGN 2

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Paper	**Country**
Puchta, H. The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J Exp Bot 56, 1-14 (2005).	DE

Transcription Activator-Like Effector Nuclease (TALEN) techniques

RESEARCH PAPERS				
Paper	**Country**	**Plant**	**Trait**	**TALEN 1,2,3**
Cermak, T. et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Research 39 (2011).	US, CN	Arabidopsis	Mutation of endogenous gene	TALEN 1
Mahfouz, M. M. et al. De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc. Natl. Acad. Sci. U. S. A. 108, 2623-2628 (2011).	SA	Tobacco	Mutation of marker gene	TALEN 1
Morbitzer, R., Elsaesser, J., Hausner, J. & Lahaye, T. Assembly of custom TALE-type DNA binding domains by modular cloning. Nucleic Acids Research 39, 5790-5799 (2011).	DE	Tobacco	Activation of marker gene	TALE-mediated activation
Morbitzer, R., Romer, P., Boch, J. & Lahaye, T. Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE)-type transcription factors. Proc. Natl. Acad. Sci. U. S. A. 107, 21617-21622 (2010).	DE	Tomato Arabidopsis Pepper	Activation of endogenous genes	TALE-mediated activation
Weber, E., Gruetzner, R., Werner, S., Engler, C. & Marillonnet, S. Assembly of Designer TAL Effectors by Golden Gate Cloning. Plos One 6 (2011).	DE	Tobacco	Activation of marker gene	TALE-mediated activation

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Paper	**Country**
Bogdanove, A. J. & Voytas, D. F. TAL Effectors: Customizable Proteins for DNA Targeting. Science 333, 1843-1846 (2011).	US
Mahfouz, M. M. & Li, L. TALE nucleases and next generation GM crops. Landes Bioscience 2, 99-103 (2011)	SA

RNA-dependent DNA methylation (RdDM)

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Paper	**Country**	**Plant**	**Trait**
Aufsatz, W., Mette, M. F., van der Winden, J., Matzke, A. J. & Matzke, M. RNA-directed DNA methylation in Arabidopsis. *Proc Natl Acad Sci U S A* **99 Suppl 4**, 16499-16506 (2002)	AT	Arabidopsis	Silencing of marker gene
Aufsatz, W., Mette, M. F., van der Winden, J., Matzke, M. & Matzke, A. J. M. HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. *Embo Journal* **21**, 6832-6841 (2002)	AT	Arabidopsis	Silencing of marker gene
Bai, S. L., Kasai, A., Yamada, K., Li, T. Z. & Harada, T. A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner. Journal of Experimental Botany 62, 4561-4570 (2011).	JP, CN	Tobacco	Silencing of marker gene
Cigan, A. M., Unger-Wallace, E. & Haug-Collet, K. Transcriptional gene silencing as a tool for uncovering gene function in maize. *Plant Journal* **43**, 929-940 (2005)	US	Maize	Male-sterility
Dalakouras, A. *et al.* A hairpin RNA construct residing in an intron efficiently triggered RNA-directed DNA methylation in tobacco. *Plant Journal* **60**, 840-851 (2009)	DE	Tobacco	Silencing of marker gene
Daxinger, L. *et al.* A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. *Embo Journal* **28**, 48-57 (2009)	AT	Arabidopsis	Silencing of marker gene
Eun, C. et al. AGO6 Functions in RNA-Mediated Transcriptional Gene Silencing in Shoot and Root Meristems in Arabidopsis thaliana. Plos One 6 (2011).	AT, UK, US	Arabidopsis	Silencing of marker gene
Fischer, U., Kuhlmann, M., Pecinka, A., Schmidt, R. & Mette, M. F. Local DNA features affect RNA-directed transcriptional gene silencing and DNA methylation. *Plant Journal* **53**, 1-10 (2008)	DE	Arabidopsis	Silencing of marker gene
Fu, X., Kohli, A., Twyman, R. M. & Christou, P. Alternative silencing effects involve distinct types of non-spreading cytosine methylation at a three-gene, single-copy transgenic locus in rice. *Molecular and General Genetics* **263**, 106-118 (2000)	UK	Rice	Silencing of marker gene
Heilersig, B., Loonen, A., Janssen, E. M., Wolters, A. M. A. & Visser, R. G. F. Efficiency of transcriptional gene silencing of GBSSI in potato depends on the promoter region that is used in an inverted repeat. *Mol. Genet. Genomics* **275**, 437-449 (2006)	NL	Potato	Modified starch content
Kanazawa, A. et al. Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants. Plant Journal 65, 156-168 (2011).	JP	1. Petunia 2.Tomato	1. Reduce flower colour 2.no ripening
Kanno, T. et al. A structural-maintenance-of-chromosomes hinge domain-containing protein is required for RNA-directed DNA methylation. Nature Genet. 40, 670-675 (2008).	AT	Arabidopsis	Silencing of marker gene
Kapoor, M. *et al.* Transgene-triggered, epigenetically regulated ectopic expression of a flower homeotic gene pMADS3 in Petunia. *Plant Journal* **43**, 649-661 (2005)	JP	Petunia	Silencing of homeotic genes
Kunz, C. *et al.* Studies on the effects of a flanking repetitive sequence on the expression of single-copy transgenes in Nicotiana sylvestris and in N-sylvestris-N-tomentosiformis hybrids. *Plant Mol.Biol.* **52**, 203-215 (2003)	AT, FR, CZ, TH, KR, AR	Tobacco	Silencing of marker gene
Lunerova-Bedrichova, J. *et al.* Trans-generation inheritance of methylation patterns in a tobacco transgene following a post-transcriptional silencing event. *Plant Journal* **54**, 1049-1062 (2008)	CZ, BE	Tobacco	Silencing of marker gene

Melnyk, C. W., Molnar, A., Bassett, A. & Baulcombe, D. C. Mobile 24 nt Small RNAs Direct Transcriptional Gene Silencing in the Root Meristems of Arabidopsis thaliana. Curr. Biol. 21, 1678-1683 (2011).	UK	Arabidopsis	Silencing of marker gene
Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *Embo Journal* **19**, 5194-5201 (2000)	AT	Tobacco	Silencing of marker gene
Mette, M. F., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. *Embo Journal* **18**, 241-248 (1999)	AT	Tobacco, Arabidopsis	Silencing of marker gene
Miki, D. & Shimamoto, K. De novo DNA methylation induced by siRNA targeted to endogenous transcribed sequences is gene-specific and OsMet1-independent in rice. *Plant Journal* **56**, 539-549 (2008)	JP	Rice	silencing of endogenous genes
Naumann, U. et al. Genetic Evidence That DNA Methyltransferase DRM2 Has a Direct Catalytic Role in RNA-Directed DNA Methylation in Arabidopsis thaliana. Genetics 187, 977-979 (2011).	AT	Arabidopsis	Silencing of marker gene
Okano, Y., Miki, D. & Shimamoto, K. Small interfering RNA (siRNA) targeting of endogenous promoters induces DNA methylation, but not necessarily gene silencing, in rice. *Plant Journal* **53**, 65-77 (2008)	JP	Rice	Silencing of marker gene
Otagaki, S., Kawai, M., Masuta, C. & Kanazawa, A. Size and positional effects of promoter RNA segments on virus-induced RNA-directed DNA methylation and transcriptional gene silencing. Epigenetics 6, 681-691 (2011).	JP	Arabidopsis	Silencing of marker gene
Shibukawa, T., Yazawa, K., Kikuchi, A. & Kamada, H. Possible involvement of DNA methylation on expression regulation of carrot LEC1 gene in its 5 '-upstream region. *Gene* **437**, 22-31 (2009)	JP	Carrot (cells)	Analysis of embryogenesis transcription factor
Sijen, T. *et al.* Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr Biol* **11**, 436-440 (2001)	NL	Petunia	Reduced flower pigmentation

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Paper	Country
Chen, X. M. Small RNAs - secrets and surprises of the genome. *Plant Journal* **61**, 941-958 (2010)	US
Chinnusamy, V. & Zhu, J. K. RNA-directed DNA methylation and demethylation in plants. *Sci. China Ser. C-Life Sci.* **52**, 331-343 (2009)	US
de Souza, A. J., Mendes, B. M. J. & Mourao, F. D. A. Gene silencing: Concepts, applications, and perspectives in woody plants. Sci. Agric. 64, 645-656 (2007).	BR
Eamens, A., Wang, M. B., Smith, N. A. & Waterhouse, P. M. RNA silencing in plants: Yesterday, today, and tomorrow. *Plant Physiology* **147**, 456-468 (2008)	AU
Huettel, B. *et al.* RNA-directed DNA methylation mediated by DRD1 and Pol IVb: A versatile pathway for transcriptional gene silencing in plants. *Biochim. Biophys. Acta-Gene Struct. Expression* **1769**, 358-374 (2007)	AT
Lavrov, S. A. & Kibanov, M. V. Noncoding RNAs and chromatin structure. *Biochemistry-Moscow* **72**, 1422-1438 (2007)	RU
Marenkova, T. V. & Deineko, E. V. Transcriptional Gene Silencing in Plants. Russ. J. Genet. 46, 511-520 (2010).	RU

Matzke, M. *et al.* Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochim. Biophys. Acta-Gene Struct. Expression* **1677**, 129-141 (2004)	AT
Matzke, M., Matzke, A. J. M. & Kooter, J. M. RNA: Guiding gene silencing. *Science* **293**, 1080-1083 (2001)	AT, NL
Muskens, M. W. M., Vissers, A. P. A., Mol, J. N. M. & Kooter, J. M. Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. *Plant Mol.Biol.* **43**, 243-260 (2000)	NL
Pickford, A. S. & Cogoni, C. RNA-mediated gene silencing. *Cellular and Molecular Life Sciences* **60**, 871-882 (2003)	IT
Shiba, H. & Takayania, S. RNA silencing systems and their relevance to allele-specific DNA methylation in plants. *Biosci. Biotechnol. Biochem.* **71**, 2632-2646 (2007)	JP
Vaucheret, H. & Fagard, M. Transcriptional gene silencing in plants: targets, inducers and regulators. *Trends in Genetics* **17**, 29-35 (2001)	FR
Verdel, A., Vavasseur, A., Le Gorrec, M. & Touat-Todeschini, L. Common themes in siRNA-mediated epigenetic silencing pathways. *Int J Dev Biol* **53**, 245-257 (2009)	FR
Wang, M. B. & Waterhouse, P. M. Application of gene silencing in plants. *Curr. Opin. Plant Biol.* **5**, 146-150 (2002)	AU
Wassenegger, M. RNA-directed DNA methylation. *Plant Mol.Biol.* **43**, 203-220 (2000)	DE

Reverse Breeding

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Paper	**Country**
Dirks, R. *et al.* Reverse breeding: a novel breeding approach based on engineered meiosis. *Plant Biotechnol J* **7**, 837-845 (2009)	AT, US, CN, NL
Lammerts Van Bueren, E. T., Verhoog, H., Tiemens-Hulscher, M., Struik, P. C. & Haring, M. A. Organic agriculture requires process rather than product evaluation of novel breeding techniques. *NJAS - Wageningen Journal of Life Sciences* **54**, 401-412 (2007)	NL
Wijnker, E. & de Jong, H. Managing meiotic recombination in plant breeding. *Trends Plant Sci* **13**, 640-646 (2008)	NL
Chan, S. W. L. Chromosome engineering: power tools for plant genetics. Trends in Biotechnology 28, 605-610 (2010).	US

Accelerated breeding through induction of early flowering

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Paper	**Country**	**Plant**
Yoo SK, Wu XL, Lee JS, Ahn JH (2011) AGAMOUS-LIKE 6 is a floral promoter that negatively regulates the FLC/MAF clade genes and positively regulates FT in Arabidopsis. Plant Journal 65:62-76.	2011	Arabidopsis

Takase T, Nishiyama Y, Tanihigashi H, Ogura Y, Miyazaki Y, Yamada Y, Kiyosue T (2011) LOV KELCH PROTEIN2 and ZEITLUPE repress Arabidopsis photoperiodic flowering under non-inductive conditions, dependent on FLAVIN-BINDING KELCH REPEAT F-BOX1. Plant Journal 67:608-621.	2011	Arabidopsis
Shulga OA, Mitiouchkina TY, Shchennikova AV, Skryabin KG, Dolgov SV (2011) Overexpression of AP1-like genes from Asteraceae induces early-flowering in transgenic Chrysanthemum plants. In Vitro Cell Dev Biol-Plant 47:553-560.	2011	Chrysanthemum plants
Ramachandran E, Bhattacharya SK, John SA, Bhattacharya PS, Abraham G (2011) Heterologous expression of Aspen PTM3, a MADS box gene in cotton. J Biotechnol 155:140-146.	2011	Cotton
Ma GY, Ning GG, Zhang W, Zhan J, Lv HY, Bao MZ (2011) Overexpression of Petunia SOC1-like Gene FBP21 in Tobacco Promotes Flowering Without Decreasing Flower or Fruit Quantity. Plant Mol Biol Rep 29:573-581.	2011	Tobacco
Li MR, Li HQ, Hu XY, Pan XP, Wu GJ (2011) Genetic transformation and overexpression of a rice Hd3a induces early flowering in Saussurea involucrata Kar. et Kir. ex Maxim. Plant Cell Tissue and Organ Culture 106:363-371.	2011	Saussurea involucrata
Zhang HL, Harry DE, Ma C, Yuceer C, Hsu CY, Vikram V, Shevchenko O, Etherington E, Strauss SH (2010) Precocious flowering in trees: the FLOWERING LOCUS T gene as a research and breeding tool in Populus. Journal of Experimental Botany 61:2549-2560.	2010	Poplar
Xu JH, Zhong XF, Zhang QZ, Li HY (2010) Overexpression of the GmGAL2 Gene Accelerates Flowering in Arabidopsis. Plant Mol Biol Rep 28:704-711.	2010	Arabidopsis
Xu H, Chen LJ, Qu LJ, Gu HY, Li DZ (2010) Functional conservation of the plant EMBRYONIC FLOWER2 gene between bamboo and Arabidopsis. Biotechnol Lett 32:1961-1968.	2010	Arabidopsis
Trankner C, Lehmann S, Hoenicka H, Hanke MV, Fladung M, Lenhardt D, Dunemann F, Gau A, Schlangen K, Malnoy M, Flachowsky H (2010) Over-expression of an FT-homologous gene of apple induces early flowering in annual and perennial plants. Planta 232:1309-1324.	2010	Arabidopsis, Apple and Poplar
Ruokolainen S, Ng YP, Broholm SK, Albert VA, Elomaa P, Teeri TH (2010) Characterization of SQUAMOSA-like genes in Gerbera hybrida, including one involved in reproductive transition. BMC Plant Biol 10.	2010	Gerbera hybrida
Li WM, Tao Y, Yao YX, Hao YJ, You CX (2010) Ectopic over-expression of two apple Flowering Locus T homologues, MdFT1 and MdFT2, reduces juvenile phase in Arabidopsis. Biol Plant 54:639-646.	2010	Arabidopsis
Du NX, Pijut PM (2010) Isolation and Characterization of an AGAMOUS Homolog from Fraxinus pennsylvanica. Plant Mol Biol Rep 28:344-351.	2010	Arabidopsis
Yasmeen A (2009) An improved protocol for the regeneration and transformation of tomato (cv Rio Grande). Acta Physiologiae Plantarum 31:1271-1277.	2009	Tomato
Spivak SG, Berdichevets IN, Yarmolinsky DG, Maneshina TV, Shpakovski GV, Kartel NA (2009) Construction and characteristics of transgenic tobacco Nicotiana tabacum L. plants expressing CYP11A1 cDNA encoding cytochrome P450(SCC). Russ J Genet 45:1067-1073.	2009	Tobacco

Sasani, S., Hemming, M.N., Oliver, S.N., Greenup, A., Tavakkol-Afshari, R., Mahfoozi, S., Poustini, K., Sharifi, H.R., Dennis, E.S., Peacock, W.J., Trevaskis, B., 2009. The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley (Hordeum vulgare). Journal of Experimental Botany 60, 2169-2178.	2009	Barley
Oliver, S.N., Finnegan, E.J., Dennis, E.S., Peacock, W.J., Trevaskis, B., 2009. Vernalization-induced flowering in cereals is associated with changes in histone methylation at the VERNALIZATION1 gene. Proc. Natl. Acad. Sci. U. S. A. 106, 8386-8391.	2009	Barley
Nakatsuka, T., Abe, Y., Kakizaki, Y., Kubota, A., Shimada, N., Nishihara, M., 2009. Over-expression of Arabidopsis FT gene reduces juvenile phase and induces early flowering in ornamental gentian plants. Euphytica 168, 113-119.	2009	Gentian plants
Mattioli, R., Falasca, G., Sabatini, S., Altamura, M.M., Costantino, P., Trovato, M., 2009. The proline biosynthetic genes P5CS1 and P5CS2 play overlapping roles in Arabidopsis flower transition but not in embryo development. Physiologia Plantarum 137, 72-85.	2009	Arabidopsis
Matsuda, N., Ikeda, K., Kurosaka, M., Takashina, T., Isuzugawa, K., Endo, T., Omura, M., 2009. Early Flowering Phenotype in Transgenic Pears (Pyrus communis L.) Expressing the CiFT Gene. J. Jpn. Soc. Hortic. Sci. 78, 410-416.	2009	Pear
Lewis, R.S., Kernodle, S.P., 2009. A method for accelerated trait conversion in plant breeding. Theoretical and Applied Genetics 118, 1499-1508.	2009	Tobacco
Xu, Y., Zhang, L., Ma, R.C., 2008. Functional characterization and mapping of two MADS box genes from peach (Prunus persica). Chinese Science Bulletin 53, 853-859.	2008	Arabidopsis
Souer, E., Rebocho, A.B., Bliek, M., Kusters, E., de Bruin, R.A.M., Koes, R., 2008. Patterning of inflorescences and flowers by the F-box protein DOUBLE TOP and the LEAFY homolog ABERRANT LEAF AND FLOWER of petunia. Plant Cell 20, 2033-2048.	2008	Petunia
Preston, J.C., Kellogg, E.A., 2008. Discrete developmental roles for temperate cereal grass VERNALIZATION1/FRUITFULL-like genes in flowering competency and the transition to flowering. Plant Physiology 146, 265-276.	2008	Oat and Wheat
Lazaro, A., Gomez-Zambrano, A., Lopez-Gonzalez, L., Pineiro, M., Jarillo, J.A., 2008. Mutations in the Arabidopsis SWC6 gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development. Journal of Experimental Botany 59, 653-666.	2008	Arabidopsis
Jin, J.B., Jin, Y.H., Lee, J., Miura, K., Yoo, C.Y., Kim, W.Y., Van Oosten, M., Hyun, Y., Somers, D.E., Lee, I., Yun, D.J., Bressan, R.A., Hasegawa, P.M., 2008. The SUMO E3 ligase, AtS1Z1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. Plant Journal 53, 530-540.	2008	Arabidopsis
Igasaki, T., Watanabe, Y., Nishiguchi, M., Kotoda, N., 2008. The FLOWERING LOCUS T/TERMINAL FLOWER 1 family in Lombardy poplar. Plant Cell Physiol. 49, 291-300.	2008	Arabidopsis
Hasan, M., Khan, A.J., Khan, S., Shah, A.H., Khan, A.R., Mirza, B., 2008. Transformation of tomato (Lycopersicon esculentum Mill.) with Arabidopsis early flowering gene Apetalai (API) through Agrobacterium infiltration of ripened fruits. Pak. J. Bot. 40, 161-173.	2008	Tomato

Fujiwara, S., Oda, A., Yoshida, R., Niinuma, K., Miyata, K., Tomozoe, Y., Tajima, T., Nakagawa, M., Hayashi, K., Coupland, G., Mizoguchi, T., 2008. Circadian Clock Proteins LHY and CCA1 Regulate SVP Protein Accumulation to Control Flowering in Arabidopsis. Plant Cell 20, 2960-2971.	2008	Arabidopsis
Dagustu, N., Fraser, P., Enfissi, E., Bramley, P., 2008. Screening for high callus induction and Agrobacterium-mediated transformation of sunflower (Helianthus Annuus L.). Biotechnol. Biotechnol. Equip. 22, 933-937.	2008	Sunflower
Zhu, L.H., Zhang, S., Larsson, S., Welander, M., 2007. Introduction of Arabidopsis gai gene caused early flowering in carnation. In: Read, P.E. (Ed.), Proceedings of the International Symposium on Plant Biotechnology: From Bench to Commercialization. International Society Horticultural Science, Leuven 1, pp. 83-88.	2007	Carnation
Wang, H., Liu, Y., Chong, K., Liu, B.Y., Ye, H.C., Li, Z.Q., Yan, F., Li, G.F., 2007. Earlier flowering induced by over-expression of CO gene does not accompany increase of artemisinin biosynthesis in Artemisia annua. Plant Biol. 9, 442-446.	2007	Artemisia annua
Southerton, S.G., 2007. Early flowering induction and Agrobacterium transformation of the hardwood tree species Eucalyptus occidentalis. Funct. Plant Biol. 34, 707-713.	2007	Eucalyptus
Mercer, K.L., Andow, D.A., Wyse, D.L., Shaw, R.G., 2007. Stress and domestication traits increase the relative fitness of crop-wild hybrids in sunflower. Ecol. Lett. 10, 383-393.	2007	Sunflower
Flachowsky, H., Peil, A., Sopanen, T., Elo, A., Hanke, V., 2007. Overexpression of BpMADS4 from silver birch (Betula pendula Roth.) induces early-flowering in apple (Malus x domestica Borkh.). Plant Breeding 126, 137-145.	2007	Apple
Flachowsky, H., Hattasch, C., Peil, A., Hanke, M.V., 2007. Transcription profiling on transgenic apple plants after over-expression of genes, which are involved in the flower development. In: Drew, R. (Ed.), Proceedings of the International Symposium on Structural and Functional Genomics of Horticultural Plants. International Society Horticultural Science, Leuven 1, pp. 215-221.	2007	Apple
Flachowsky, H., Hanke, M.V., Elo, A., Sopanen, T., 2007. BpMADS4 - a MADS box gene of birch induces flowers on transgenic apple plants in vitro. In: Litz, R.E., Scorza, R. (Eds.), Proceedings of the International Symposium on Biotechnology of Temperate Fruit Crops and Tropical Species. International Society Horticultural Science, Leuven 1, pp. 307-312.	2007	Apple
Dunfield, K., Srivastava, S., Shah, S., Kav, N.N.V., 2007. Constitutive expression of ABR17 cDNA enhances germination and promotes early flowering in Brassica napus. Plant Science 173, 521-532.	2007	Oilseed rape
Domagalska, M.A., Schomburg, F.M., Amasino, R.M., Vierstra, R.D., Nagy, F., Davis, S.J., 2007. Attenuation of brassinosteroid signaling enhances FLC expression and delays flowering. Development 134, 2841-2850.	2007	Arabidopsis
Schonrock, N., Bouveret, R., Leroy, O., Borghi, L., Kohler, C., Gruissem, W., Hennig, L., 2006. Polycomb-group proteins repress the floral activator AGL19 in the FLC-independent vernalization pathway. Genes Dev. 20, 1667-1678.	2006	Arabidopsis
Schlappi, M.R., 2006. FRIGIDA LIKE 2 is a functional allele in Landsberg erecta and compensates for a nonsense allele of FRIGIDA LIKE 1. Plant Physiology 142, 1728-1738.	2006	Arabidopsis

Kater, M.M., Dreni, L., Colombo, L., 2006. Functional conservation of MADS-box factors controlling floral organ identity in rice and Arabidopsis. Journal of Experimental Botany 57, 3433-3444.	2006	Arabidopsis and Rice
Hoenicka, H., Nowitzki, O., Debener, T., Fladung, M., 2006. Faster evaluation of induced floral sterility in transgenic early flowering poplar. Silvae Genet. 55, 285-291.	2006	Poplar
Fernando, D.D., Zhang, S.L., 2006. Constitutive expression of the SAP1 gene from willow (Salix discolor) causes early flowering in Arabidopsis thaliana. Dev. Genes Evol. 216, 19-28.	2006	Arabidopsis
Fang, Q.Y., Xu, Z.K., Song, R.T., 2006. Cloning, characterization and genetic engineering of FLC homolog in Thellungiella halophila. Biochemical and Biophysical Research Communications 347, 707-714.	2006	Thellungiella halophila
Chaidamsari, T., Samanhudi, Sugiarti, H., Santoso, D., Angenent, G.C., de Maagd, R.A., 2006. Isolation and characterization of an AGAMOUS homologue from cocoa. Plant Science 170, 968-975.	2006	Arabidopsis
Bouveret, R., Schonrock, N., Gruissem, W., Hennig, L., 2006. Regulation of flowering time by Arabidopsis MSI1. Development 133, 1693-1702.	2006	Arabidopsis
Kotoda, N., Wada, M., 2005. MdTFL1, a TFL1-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic Arabidopsis. Plant Science 168, 95-104.	2005	Arabidopsis
Endo, T., Shimada, T., Fujii, H., Kobayashi, Y., Araki, T., Omura, M., 2005. Ectopic expression of an FT homolog from Citrus confers an early flowering phenotype on trifoliate orange (Poncirus trifoliata L. Raf.). Transgenic Res. 14, 703-712.	2005	Trifoliate orange
Chandler, J., Corbesier, L., Spielmann, P., Dettendorfer, J., Stahl, D., Apel, K., Melzer, S., 2005. Modulating flowering time and prevention of pod shatter in oilseed rape. Mol. Breed. 15, 87-94.	2005	Oilseed rape
Yoo, S.Y., Kardailsky, I., Lee, J.S., Weigel, D., Ahn, J.H., 2004. Acceleration of flowering by overexpression of MFT (MOTHER OF FT AND TFL1). Mol. Cells 17, 95-101.	2004	Arabidopsis
Watson, J.M., Brill, E.M., 2004. Eucalyptus grandis has at least two functional SOC1-like floral activator genes. Funct. Plant Biol. 31, 225-234.	2004	Arabidopsis
Noh, Y.S., Bizzell, C.M., Noh, B., Schomburg, F.M., Amasino, R.M., 2004. EARLY FLOWERING 5 acts as a floral repressor in Arabidopsis. Plant Journal 38, 664-672.	2004	Arabidopsis
Meilan, R., Sabatti, M., Ma, C.P., Kuzminsky, E., 2004. An early-flowering genotype of Populus. J. Plant Biol. 47, 52-56.	2004	Poplar
Martin, J., Storgaard, M., Andersen, C.H., Nielsen, K.K., 2004. Photoperiodic regulation of flowering in perennial ryegrass involving a CONSTANS-like homolog. Plant Mol.Biol. 56, 159-169.	2004	Perennial ryegrass
Lemmetyinen, J., Hassinen, M., Elo, A., Porali, I., Keinonen, K., Makela, H., Sopanen, T., 2004. Functional characterization of SEPALLATA3 and AGAMOUS orthologues in silver birch. Physiologia Plantarum 121, 149-162.	2004	Silver birch
Fornara, F., Parenicova, L., Falasca, G., Pelucchi, N., Masiero, S., Ciannamea, S., Lopez-Dee, Z., Altamura, M.M., Colombo, L., Kater, M.M., 2004. Functional characterization of OsMADS18, a member of the AP1/SQUA subfamily of MADS box genes. Plant Physiology 135, 2207-2219.	2004	Rice and Arabidopsis

Ferrario, S., Busscher, J., Franken, J., Gerats, T., Vandenbussche, M., Angenent, G.C., Immink, R.G.H., 2004. Ectopic expression of the petunia MADS box gene UNSHAVEN accelerates flowering and confers leaf-like characteristics to floral organs in a dominant-negative manner. Plant Cell 16, 1490-1505.	2004	Petunia and Arabidopsis
Tadege, M., Sheldon, C.C., Helliwell, C.A., Upadhyaya, N.M., Dennis, E.S., Peacock, W.J., 2003. Reciprocal control of flowering time by OsSOC1 in transgenic Arabidopsis and by FLC in transgenic rice. Plant Biotechnology Journal 1, 361-369.	2003	Rice and Arabidopsis
Skinner, J.S., Meilan, R., Ma, C.P., Strauss, S.H., 2003. The Populus PTD promoter imparts floral-predominant expression and enables high levels of floral-organ ablation in Populus, Nicotiana and Arabidopsis. Mol. Breed. 12, 119-132.	2003	Poplar, Tobacco and Arabidopsis
Wada, M., Cao, Q.F., Kotoda, N., Soejima, J., Masuda, T., 2002. Apple has two orthologues of FLORICAULA/LEAFY involved in flowering. Plant Mol.Biol. 49, 567-577.	2002	Arabidopsis
Yoshida, N., Yanai, Y., Chen, L.J., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R., Takahashi, S., 2001. EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. Plant Cell 13, 2471-2481	2001	Arabidopsis
Schomburg, F.M., Patton, D.A., Meinke, D.W., Amasino, R.M., 2001. FPA, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. Plant Cell 13, 1427-1436.	2001	Arabidopsis
Pena, L., Martin-Trillo, M., Juarez, J., Pina, J.A., Navarro, L., Martinez-Zapater, J.M., 2001. Constitutive expression of Arabidopsis LEAFY or APETALA1 genes in citrus reduces their generation time. Nature Biotechnology 19, 263-267.	2001	Citrus
Mercuri, A., Bruna, S., De Benedetti, L., Burchi, G., Schiva, T., 2001. Modification of plant architecture in Limonium spp. induced by rol genes. Plant Cell Tissue and Organ Culture 65, 247-253.	2001	Limonium
Kim, S., Kim, S.R., An, C.S., Hong, Y.N., Lee, K.W., 2001. Constitutive expression of rice MADS box gene using seed explants in hot pepper (Capsicum annuum L.). Mol. Cells 12, 221-226.	2001	Hot pepper
Elo, A., Lemmetyinen, J., Turunen, M.L., Tikka, L., Sopanen, T., 2001. Three MADS-box genes similar to APETALA1 and FRUITFULL from silver birch (Betula pendula). Physiologia Plantarum 112, 95-103.	2001	Tobacco
Berbel, A., Navarro, C., Ferrandiz, C., Canas, L.A., Madueno, F., Beltran, J.P., 2001. Analysis of PEAM4, the pea AP1 functional homologue, supports a model for AP1-like genes controlling both floral meristem and floral organ identity in different plant species. Plant Journal 25, 441-451.	2001	Tobacco and Arabidopsis
Jeon, J.S., Lee, S., Jung, K.H., Yang, W.S., Yi, G.H., Oh, B.G., An, G.H., 2000. Production of transgenic rice plants showing reduced heading date and plant height by ectopic expression of rice MADS-box genes. Mol. Breed. 6, 581-592.	2000	Rice
He, Z.H., Zhu, Q., Dabi, T., Li, D.B., Weigel, D., Lamb, C., 2000. Transformation of rice with the Arabidopsis floral regulator LEAFY causes early heading. Transgenic Res. 9, 223-227.	2000	Rice
Yao, J.L., Cohen, D., van den Brink, R., Morris, B., 1999. Assessment of expression and inheritance patterns of three transgenes with the aid of techniques for promoting rapid flowering of transgenic apple trees. Plant Cell Reports 18, 727-732.	1999	Apple

Page, T., Macknight, R., Yang, C.H., Dean, C., 1999. Genetic interactions of the Arabidopsis flowering time gene FCA, with genes regulating floral initiation. Plant Journal 17, 231-239.	1999	Arabidopsis
Giovannini, A., Zottini, M., Morreale, G., Spena, A., Allavena, A., 1999. Ornamental traits modification by rol genes in Osteospermum ecklonis transformed with Agrobacterium tumefaciens. In Vitro Cell. Dev. Biol.-Plant 35, 70-75.	1999	Osteospermum ecklonis
Lemmetyinen, J., Keinonen-Mettala, K., Lannenpaa, M., von Weissenberg, K., Sopanen, T., 1998. Activity of the CaMV 35S promoter in various parts of transgenic early flowering birch clones. Plant Cell Reports 18, 243-248.	1998	Birch
Finnegan, E.J., Genger, R.K., Kovac, K., Peacock, W.J., Dennis, E.S., 1998. DNA methylation and the promotion of flowering by vernalization. Proc. Natl. Acad. Sci. U. S. A. 95, 5824-5829.	1998	Arabidopsis
Kania, T., Russenberger, D., Peng, S., Apel, K., Melzer, S., 1997. FPF1 promotes flowering in Arabidopsis. Plant Cell 9, 1327-1338.	1997	Arabidopsis
Kang, H.G., Jang, S., Chung, J.E., Cho, Y.G., An, G., 1997. Characterization of two rice MADS box genes that control flowering time. Mol. Cells 7, 559-566.	1997	Tobacco

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Paper	**Country**
Flachowsky, H., Hanke, M.V., Peil, A., Strauss, S.H., Fladung, M., 2009. A review on transgenic approaches to accelerate breeding of woody plants. Plant Breeding 128, 217-226.	2009
Trevaskis, B., Hemming, M.N., Dennis, E.S., Peacock, W.J., 2007. The molecular basis of vernalization-induced flowering in cereals. Trends in Plant Science 12, 352-357.	2007
Roux, F., Touzet, P., Cuguen, J., Le Corre, V., 2006. How to be early flowering: an evolutionary perspective. Trends in Plant Science 11, 375-381.	2006
Bernier, G., Perilleux, C., 2005. A physiological overview of the genetics of flowering time control. Plant Biotechnology Journal 3, 3-16.	2005
Sung, Z.R., Chen, L.J., Moon, Y.H., Lertpiriyapong, K., 2003. Mechanisms of floral repression in Arabidopsis. Curr. Opin. Plant Biol. 6, 29-35.	2003

Cisgenesis and Intragenesis

RESEARCH PAPERS			
Paper	**Country**	**Plant**	**Trait**
Belfanti, E. *et al.* The HcrVf2 gene from a wild apple confers scab resistance to a transgenic cultivated variety. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 886-890 (2004)	IT, CH	Apple	Fungal resistance
Benjamin, I., Kenigsbuch, D., Galperin, M., Abrameto, J. & Cohen, Y. Cisgenic melons over expressing glyoxylate-aminotransferase are resistant to downy mildew. *European Journal of Plant Pathology* **125**, 355-365 (2009)	IL	Melon	Fungal resistance

Dhekney, S. A., Li, Z. J. T. & Gray, D. J. Grapevines engineered to express cisgenic Vitis vinifera thaumatin-like protein exhibit fungal disease resistance. In Vitro Cell. Dev. Biol.-Plant 47, 458-466 (2011).	US	Grapevine	Fungal resistance
Han, K. M. et al. Gibberellin-associated cisgenes modify growth, stature and wood properties in Populus. Plant Biotechnology Journal 9, 162-178 (2011).	US	Poplar	Modified tree structure
Joshi, S. G. et al. Functional analysis and expression profiling of HcrVf1 and HcrVf2 for development of scab resistant cisgenic and intragenic apples. Plant Mol.Biol. 75, 579-591 (2011).	NL	Apple	Fungal resistance
Kuhl, J. C., Zarka, K., Coombs, J., Kirk, W. W. & Douches, D. S. Late Blight Resistance of RB Transgenic Potato Lines. *Journal of the Americal Society for Horticultural Science* **132**, 783-789 (2007)	US	Potato	Fungal resistance
Park, T. H., Foster, S., Brigneti, G. & Jones, J. D. G. Two distinct potato late blight resistance genes from Solanum berthaultii are located on chromosome 10. *Euphytica* **165**, 269-278 (2009)	UK, KR	Potato	Fungal resistance
Rommens, C. M. *et al.* Plant-derived transfer DNAs. *Plant Physiol* **139**, 1338-1349 (2005)	US	Potato	Black spot bruise tolerance
Rommens, C. M. *et al.* Crop Improvement through Modification of the Plant's Own Genome. *Plant Physiology* **135**, 421-431 (2004)	US	Tobacco	Marker genes
Rommens, C. M., Yan, H., Swords, K., Richael, C. & Ye, J. S. Low-acrylamide French fries and potato chips. *Plant Biotechnology Journal* **6**, 843-853 (2008)	US	Potato	Lower acrylamide levels
Rommens, C. M., Ye, J., Richael, C. & Swords, K. Improving potato storage and processing characteristics through all-native DNA transformation. *J Agric Food Chem* **54**, 9882-9887 (2006)	US	Potato	Lower acrylamide levels
Schaart, J. G., Tinnenbroek-Capel, I. E. M. & Krens, F. A. Isolation and characterization of strong gene regulatory sequences from apple, Malus x domestica. Tree Genetics & Genomes 7, 135-142 (2011).	NL	Apple	Test of cisgenic promoters and terminators
Silfverberg-Dilworth, E. *et al.* Identification of functional apple scab resistance gene promoters. *Theoretical and Applied Genetics* **110**, 1119-1126 (2005)	IT, CH	Apple	Fungal resistance
Szankowski, I. *et al.* Highly scab-resistant transgenic apple lines achieved by introgression of HcrVf2 controlled by different native promoter lengths. *Tree Genetics & Genomes* **5**, 349-358 (2009)	DE, CH, IT, BR	Apple	Fungal resistance
Vanblaere, T. et al. The development of a cisgenic apple plant. J. Biotechnol. 154, 304-311 (2011).	CH, NL, DE	Apple	Fungal resistance
Weeks, J. T., Ye, J. S. & Rommens, C. M. Development of an in planta method for transformation of alfalfa (Medicago sativa). Transgenic Res. 17, 587-597 (2008).	US	Alfalfa	Improved lignin content

REVIEW PAPERS

Paper	Country
Akhond, M. A. Y. & Machray, G. C. Biotech crops: technologies, achievements and prospects. *Euphytica* **166**, 47-59 (2009)	BD, UK
Bhatti, S. & Jha, G. Current trends and future prospects of biotechnological interventions through tissue culture in apple. Plant Cell Reports 29, 1215-1225 (2010).	IN

Bruening, G. in Annual Review of Phytopathology, Vol 49 Vol. 49 Annual Review of Phytopathology(eds N. K. VanAlfen, G. Bruening, & J. E. Leach) 1-16 (Annual Reviews, 2011).	US
Conner, A. *et al.* Intragenic vectors for gene transfer without foreign DNA. *Euphytica* **154**, 341-353 (2007)	NZ, NL
Haverkort, A. *et al.* Societal Costs of Late Blight in Potato and Prospects of Durable Resistance Through Cisgenic Modification. *Potato Research* **51**, 47-57 (2008)	NL
Jacobsen, E. & Nataraja, K. N. Cisgenics - Facilitating the second green revolution in India by improved traditional plant breeding. *Current Science* **94**, 1365-1366 (2008)	NL, IN
Jacobsen, E. & Schouten, H. Cisgenesis, a New Tool for Traditional Plant Breeding, Should be Exempted from the Regulation on Genetically Modified Organisms in a Step by Step Approach. *Potato Research* **51**, 75-88 (2008)	NL
Jacobsen, E. & Schouten, H. J. Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. *Trends Biotechnol* **25**, 219-223 (2007)	NL
Jacobsen, E. & Schouten, H. J. Cisgenesis: an important sub-invention for traditional plant breeding companies. *Euphytica* **170**, 235-247 (2009)	NL
Kok, E. J., Keijer, J., Kleter, G. A. & Kuiper, H. A. Comparative safety assessment of plant-derived foods. *Regul Toxicol Pharmacol* **50**, 98-113 (2008)	NL
Myskja, B. K. The moral difference between intragenic and transgenic modification of plants. *Journal of Agricultural & Environmental Ethics* **19**, 225-238 (2006)	NO
Nielsen, K. M. Transgenic organisms--time for conceptual diversification? *Nat Biotechnol* **21**, 227-228 (2003)	NO
Park, T. H., Vleeshouwers, V., Jacobsen, E., van der Vossen, E. & Visser, R. G. F. Molecular breeding for resistance to Phytophthora infestans (Mont.) de Bary in potato (Solanum tuberosum L.): a perspective of cisgenesis. *Plant Breeding* **128**, 109-117 (2009)	NL
Polanco, V., Paredes, M., Becerra, V. & Pérez, E. Advances in apple transformation technology to confer resistance to fungal diseases in apple crops: a Chilean perspective. Chilean Journal of Agricultural Research 70(2), 279-308 (2010)	CL
Rommens, C. M. All-native DNA transformation: a new approach to plant genetic engineering. *Trends Plant Sci* **9**, 457-464 (2004)	US
Rommens, C. M. Intragenic crop improvement: combining the benefits of traditional breeding and genetic engineering. *J Agric Food Chem* **55**, 4281-4288 (2007)	US
Rommens, C. M. The need for professional guidelines in plant breeding. *Trends in Plant Science* **13**, 261-263 (2008)	US
Rommens, C. M. Barriers and paths to market for genetically engineered crops. *Plant Biotechnology Journal* **8**, 101-111 (2010)	US
Rommens, C. M., Haring, M. A., Swords, K., Davies, H. V. & Belknap, W. R. The intragenic approach as a new extension to traditional plant breeding. *Trends Plant Sci* **12**, 397-403 (2007)	US, NL, UK
Rosellini, D. Selectable marker genes from plants: reliability and potential. In Vitro Cell. Dev. Biol.-Plant 47, 222-233 (2011).	IT
Russell, A. W. & Sparrow, R. The case for regulating intragenic GMOS. *Journal of Agricultural & Environmental Ethics* **21**, 153-181 (2008)	AU
Schouten, H. J. & Jacobsen, E. Are mutations in genetically modified plants dangerous? *J. Biomed. Biotechnol.* (2007)	NL

Schouten, H. J. & Jacobsen, E. Cisgenesis and intragenesis, sisters in innovative plant breeding. *Trends Plant Sci* **13**, 260-261; author reply 261-263 (2008)	NL
Schouten, H. J., Krens, F. A. & Jacobsen, E. Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Rep* **7**, 750-753 (2006)	NL
Schouten, H. J., Krens, F. A. & Jacobsen, E. Do cisgenic plants warrant less stringent oversight? *Nat Biotechnol* **24**, 753 (2006)	NL
Schouten, H. J. *et al.* Cisgenesis is a promising approach for fast, acceptable and safe breeding of pip fruit. *Acta Hort.* **814**, 199-204 (2009)	NZ, NL
Schubert, D. & Williams, D. 'Cisgenic' as a product designation. *Nat Biotech* **24**, 1327-1329 (2006)	US

Grafting non-GM varieties onto GM rootstocks

RESEARCH PAPERS

Paper	Country	Plant	Trait
Aguero, C. B. *et al.* Evaluation of tolerance to Pierce's disease and Botrytis in transgenic plants of Vitis vinifera L. expressing the pear PGIP gene. *Mol. Plant Pathol.* **6**, 43-51 (2005)	US	Grapevine	Resistance vs bacteria and fungi
Conrath, U. *et al.* Enhanced resistance to Phytophthora infestans and Alternaria solani in leaves and tubers, respectively, of potato plants with decreased activity of the plastidic ATP/ADP transporter. *Planta* **217**, 75-83 (2003)	UK, DE	Potato	Fungal resistance
Derrick, P. M. & Barker, H. Short and long distance spread of potato leafroll luteovirus: Effects of host genes and transgenes conferring resistance to virus accumulation in potato. *J. Gen. Virol.* **78**, 243-251 (1997)	UK	Potato	Virus resistance
Gal-On, A. *et al.* Transgenic cucumbers harboring the 54-kDa putative gene of Cucumber fruit mottle mosaic tobamovirus are highly resistant to viral infection and protect non-transgenic scions from soil infection. *Transgenic Res* **14**, 81-93 (2005)	IL, KR	Cucumber	Virus resistance
Han, J. S., Park, S., Shigaki, T., Hirschi, K. D. & Kim, C. K. Improved watermelon quality using bottle gourd rootstock expressing a Ca2+/H+ antiporter. *Mol. Breed.* **24**, 201-211 (2009)	US, KR	Watermelon	Robust growth
Lambert, C. & Tepfer, D. Use of *Agrobacterium rhizogenes* to create chimeric apple-trees through genetic grafting. *Bio-Technology* **9**, 80-83 (1991)	FR	Apple	Rooting ability
Mackenzie, D. J., Tremaine, J. H. & McPherson, J. Genetically engineered resistance to potato virus-S in potato cultivar Russet Burbank. *Molecular Plant-Microbe Interactions* **4**, 95-102 (1991)	CA	Potato	Virus resistance
Maki-Valkama, T. *et al.* High level of resistance to potato virus Y by expressing P1 sequence in antisense orientation in transgenic potato. *Mol. Breed.* **6**, 95-104 (2000)	SE, FI	Potato	Virus resistance
McGurl, B., Orozcocardenas, M., Pearce, G. & Ryan, C. A. Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase-inhibitor synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9799-9802 (1994)	US	Tomato	Resistance vs insects

Mitani, N., Kobayashi, S., Nishizawa, Y., Takeshi, K. & Matsumoto, R. Transformation of trifoliate orange with rice chitinase gene and the use of the transformed plant as a rootstock. *Sci. Hortic.* **108**, 439-441 (2006)	JP	Orange	Fungal resistance
Nagel, A. K., Kalariya, H. & Schnabel, G. The Gastrodia Antifungal Protein (GAFP-1) and Its Transcript Are Absent from Scions of Chimeric-grafted Plum. *Hortscience* **45**, 188-192 (2010)	US	Plum	Resistance vs fungi and nematodes
Park, S. M. *et al.* Transgenic watermelon rootstock resistant to CGMMV (cucumber green mottle mosaic virus) infection. *Plant Cell Rep* **24**, 350-356 (2005)	KR	Watermelon	Virus resistance
Smolka, A., Li, X. Y., Heikelt, C., Welander, M. & Zhu, L. H. Effects of transgenic rootstocks on growth and development of non-transgenic scion cultivars in apple. Transgenic Res. 19, 933-948 (2010).	SE	Apple	Rooting ability
Vahdati, K. *et al.* Rooting and other characteristics of a transgenic walnut hybrid (Juglans hindsii x J. regia) rootstock expressing rolABC. *J. Am. Soc. Hortic. Sci.* **127**, 724-728 (2002)	US	Walnut	Rooting ability
Van den Boogaart, T., Maule, A. J., Davies, J. W. & Lomonossoff, G. P. Sources of target specificity associated with the recovery against Pea seed-borne mosaic virus infection mediated by RNA silencing in pea. *Mol. Plant Pathol.* **5**, 37-43 (2004)	UK	Pea	Virus resistance
van der Salm, T. P. M. et al. Stimulation of scion bud release by rol gene transformed rootstocks of Rosa hybrida L. Journal of Experimental Botany 49, 847-852 (1998).	NL	Rose	Rooting ability
Vigne, E., Komar, V. & Fuchs, M. Field safety assessment of recombination in transgenic grapevines expressing the coat protein gene of Grapevine fanleaf virus. Transgenic Res. 13, 165-179 (2004).	FR	Grapevine	Virus resistance
Visser, P. B., Keizer, P., Van der Salm, T. P. M. & De Jong, J. Rootstocks transformed with rol A, B, C genes enhance rose flower production. Biotechnol. Biotechnol. Equip. 14, 63-70 (2000)	NL	Rose	Rooting ability
Youk, E. S. et al. A framework for molecular genetic assessment of a transgenic watermelon rootstock line. Plant Science 176, 805-811 (2009).	KR	Watermelon	Virus resistance
Zhang, H. L. et al. Precocious flowering in trees: the FLOWERING LOCUS T gene as a research and breeding tool in Populus. Journal of Experimental Botany 61, 2549-2560 (2010).	CN, US	Poplar	Early flowering
Zhu, L. H. & Welander, M. Growth characteristics of apple cultivar Gravenstein plants grafted onto the transformed rootstock M26 with rolA and rolB genes under non-limiting nutrient conditions. Plant Science 147, 75-80 (1999).	SE	Apple	Rooting ability

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Paper	Country
Lough, T. J. & Lucas, W. J. Integrative plant biology: Role of phloem long-distance macromolecular trafficking. *Annual Review of Plant Biology* **57**, 203-232 (2006)	US, NZ
van der Salm, T. P. M., TenCate, C. H. H. & Dons, H. J. M. Prospects for applications of rol genes for crop improvement. *Plant Mol. Biol. Rep.* **14**, 207-228 (1996)	NL
Walker, G. E. & Stirling, G. R. Plant-parasitic nematodes in Australian viticulture: key pests, current management practices and opportunities for future improvements. Austral. Plant Pathol. 37, 268-278 (2008).	AU

Annex II. Patent search results on New Plant Breeding Techniques

Oligonucleotide directed mutagenesis (ODM)

Patent	Country	Plant	Trait
Andrews, W. H., Morser, M. J. & Vilander, L. R. Novel Mutagenesis Methods And Compositions, WO/93/01282. Berlex Lab (US) (1991)	US	Plants in general	Targeted mutation
AndrUS, A. & Kuimelis, R. G. Improved Chimeric Oligonucleotide Vectors, WO/98/39353 Perkin Elmer Corp (US) (1997)	US	Plants in general	Targeted mutation
Arntzen, C. J., Kipp, P. B., Kumar, R. & May, G. D. USe Of Mixed Duplex Oligonucleotides To Effect Localized Genetic Changes In Plants, WO/99/07865. Kimeagen Inc (US) (1997)	US	Maize, wheat, rice, lettuce, potato, tomato, rapeseed, soybean, cotton	Targeted mutation: herbicide tolerance and resistance to various substances
Badur, R. & Reiss, B. Method For Producing Recombinant Organisms, WO/2004/085644. Basf Plant Science Gmbh (DE) (2003)	DE	Higher plants	Targeted mutation: decrease chromatin assembly capacity
Baszczynski, C. L. Et Al. Targeted Manipulation Of Herbicide-Resistance Genes In Plants, WO/99/25853. Pioneer Hi Bred Int (US) (1997)	US	Maize	Targeted mutation: herbicide tolerance
Beetham, P., Avissar, P. & Walker, K. Compositions And Methods For Plant Genetic Modification, WO/01/25460. Valigen Inc (US) (1999)	US	Brassicaceae family	Targeted mutation
Beetham, P. R., Avissar, P. L., Walker, K. A. & Metz, R. A. Methods of making non-transgenic herbicide resistant plants. US/6870075. Valigen US Inc (US) (1999).	US	Group of plants	Targeted mutation: herbicide tolerance
Beethan, P. et al. Herbicide-Resistant AHAS-mutants and Methods of use. WO/2010/036771. BASF Agrochemical Products BV (NL) (2008).	NL	Brassica plants	Targeted mutation: herbicide tolerance
Brachman, E., Ferrara, L., Kmiec, E. B. & Parekh-Olmedo, H. Methods And Kits To Increase The Efficiency Of Oligonucleotide-Directed Nucleic Acid Sequence Alteration, WO/2005/108622. Univ Delaware (US) (2004)	US	Plants in general	Increase efficiency of mutagenesis
Bundock, P. Targeted Nucleotide Exchange With Improved Modified Oligonucleotides, WO/2009/002150. Keygene Nv (NL) (2007)	NL	Plants in general	Targeted mutation: herbicide tolerance
Bundock, P. *Et Al.* Improved Targeted Nucleotide Exchange With Lna Modified Oligonucleotides, Ep/2002/001. Keygene Nv (NL) (2005)	NL	Plants in general	Targeted mutation
Bundock, P., De Both, M., Theodoor, J. & Lhuissier, F. An Improved Mutagenesis Method Using Polyethylene Glycol Mediated Introduction Of Mutagenic Nucleobases Into Plant Protoplasts, WO/2009/082190. Keygene Nv (NL) (2007)	NL	Plants protoplasts	Targeted mutation
Bundock, P. & Lhuissier, F. Targeted Alteration of DNA. WO/2012/074385. Keygene NV (NL) (2010).	NL	Plants in general	Targeted mutation
De Both, M. T. J. & Furukawa, T. Targeted Alteration of DNA with Oligonucleotides. WO/2012/074386. Keygene NV (NL) (2010).	NL	Plants in general	Targeted mutation
De Wit, J. P. C., Van Dun, C. M. P. & Schut, J. W. Reduced susceptibility towards pathogens, in particular oomycetes, such as downy mildew in lettuce and spinach. WO/2005/124108. Rijk Zwaan Zaadteelt en Zaadha (NL) (2004).	NL	Lettuce and Spinach	Targeted mutation: fungal resistance
Gamper, H. B., Kimiec, E. & Bartlett, R. Binary Hybrid Mutational Vectors, WO/01/94610. Univ Jefferson (US), Univ Miami (US) (2000)	US	Plants in general	Targeted mutation
Gocal, G., Avissar, P., Knuth, M., Beetham, P. & Walker, K. Non-Transgenic Herbicide Resistant Plants, WO/03/013226. Cibus Genetics (US) (2001)	US	Maize, rapeseed, Petunia + group of crop plants	Targeted mutation: herbicide tolerance
Gocal, G. F. W., Knuth, M. E. & Beetham, P. R. Epsps Mutants, WO/2007/084294. Cibus Llc (US) (2006)	US	Maize, rapeseed, Petunia + group of crop plants	Targeted mutation: herbicide tolerance

Goff, S. A. Locked Nucleic Acid Containing Heteropolymers And Related Methods, US/2006/117410. Syngenta ParticipatioUS Ag (CH) (2001)	US	Maize, tobacco	Targeted mutation: herbicide tolerance
Hawkes, T. R., Greenland, A. J. & Evans, I. J. Methods Of In Situ Modification Of Plant Genes, WO/98/54330. Zeneca Ltd (GB) (1997)	GB	Group of crop plants	Targeted mutation: herbicide tolerance
Kmiec, E. B. Chimeric Mutational Vectors Having Non-Natural Nucleotides, WO/97/48714. Univ Jefferson (US), Univ Miami (US) (1996)	US	Plants in general	Targeted mutation
Kmiec, E. B., Gamper, H. B. & Rice, M. C. Targeted Chromosomal Genomic Alterations With Modified Single Stranded Oligonucleotides, Ep/1268768. University Of Delaware (US) (2000)	US	Plants in general	Targeted mutation
Kmiec, E. B., Gamper, H. B., Rice, M. C. & Kim, J. Targeted Chromosomal Genomic Alterations In Plants Using Modified Single Stranded Oligonucleotides, US/2003/236208. Univ Delaware (US) (2000)	US	Plants in general	Targeted mutation
Kmiec, E. B., Gamper, H. B., Rice, M. & Liu, L. Method for Enhancing Targeted Gene Alteration Using Oligonucleotides. WO/2001/73002. University of Delaware (US) (2000).	US	Plants in general	Targeted mutation
Kmiec, E. B., Rice, M. C. & Liu, L. Cmposition and Methods for Enhancing Oligonucleotide-directed Nucleic Acid Sequence Alteration. WO/2003/027265. University of Delaware (US) (2001).	US	Plants in general	Targeted mutation
Kmiec, E. B., Parekh-Olmedo, H. & Brachman, E. E. Methods, Compositions, And Kits For Enhancing Oligonucleotide-Mediated Nucleic Acid Sequence Alteration USing Compositions Comprising A Histone Deacetylase Inhibitor, Lambda Phage Beta Protein, Or Hydroxyurea, WO/03/075856. Univ Delaware (US) (2002)	US	Plants in general	Targeted mutation
Knuth, M. E., Beetham, P. R., Walker, K. A. & Gocal, G. F. W. Fatty acid blends and uses therefor. WO/2008/002643. Cibus LLC (US) (2006).	US	Oilseed rape, Soybean, Maize, Cotton	Targeted mutation: modified fatty acids content
Mahajan, P. B. & Kannan, P. Targeted Manipulation Of Genes In Plants, WO/03/076574. Pioneer Hi Bred Int (US) (2002)	US	Plants in general	Targeted mutation: herbicide tolerance + disease resistance
May, G. D., Kmiec, E. B. & Rice, M. C. Plant Gene Targeting Using Oligonucleotides, WO/01/87914. Univ Delaware (US) (2000)	US	Plants in general	Targeted mutation
Metz, R., Frank, B. & Walther, D. Single-stranded oligodeoxynucleotide mutational vectors. WO/2001/15740. Valigen US Inc (US) (1999)	US	Plants in general	Targeted mutation
Prokopishyn, N. L. Short Fragment HomologoUS Recombination To Effect Targeted Genetic Alterations In Plants, WO/03/062425. Prokopishyn Nicole Lesley (US) (2002)	US	Plants in general	Targeted mutation
Rainey-Wittich, D. Y., De Both, M., Theodoor, J. & Bundock, P. Method And Means For Targeted Nucleotide Exchange, WO/2007/037676. Keygene Nv (NL) (2005)	NL	Plants in general	Targeted mutation
Schopke, C., Gocal, G. F. W., Walker, K. & Beetham, P. R. Mutated Acetohydroxyacid Synthase Genes In Brassica, WO/2009/046334. CibUS Llc (US) (2007)	US	Rapeseed	Targeted mutation: herbicide tolerance
Sundaresan, V. & Rajani, S. Dehiscence Gene And Methods For Regulating Dehiscence, WO/01/59122. Inst Of Molecular Agrobiology (SG) (2000)	SG	Fruit plants	Targeted mutation: dehiscence prevention

Zinc finger nuclease (ZFN) techniques (ZFN1,2,3)

Patent	Country	Plant	Trait	ZFN 1,2,3
Ainley, W. M. et al. Engineered landing pads for gene targeting in plants. WO/2011/091317. Dow Agrosciences LLC (US) (2010).	US	Monocots and dicots, including oilseed rape	Targeted insertion	ZFN 3
Ainley, W. M., Murray, M. G., Urnov, F. & Zeitler, B. Targeted genomic alteration. WO/2011/090804. Dow Agrosciences LLC (US), Sangamo Biosciences Inc (US), (2010).	US	Plants in general	Targeted insertion	ZFN 3
Biesgen, C. Methods For The Transformation Of Vegetal Plastids, WO/03/054189. Sungene Gmbh & Co. Kgaa - Basf (DE) (2001)	DE	Arabidopsis + crop plants	Targeted insertion	ZFN 3
Bundock, P. & De Both, M. T. J. dsRNA for improved genetic modification of plant DNA. WO/2011/078662. Keygene NV (NL) (2009)	NL	Group of dicots	Targeted mutation	ZFN 1
Bundock, P., De Both, M. T. J. & Lhuissier, F. A device for dispensing a substance. WO/2011/078665. Keygene (NL) (2009).	NL	Plants in general	Targeted mutation	ZFN 1
Butler, H. et al. Targeted integration into the Zp15 locus, WO/2010/077319. Dow AgroSciences LLC (US), Sangamo BioSciences Inc (US), (2009).	US	Maize	Targeted insertion: multiple traits	ZFN 3
Cai, Q. C. *Et Al.* Optimized Non-Canonical Zinc Finger Proteins, WO/2008/076290 Sangamo Biosciences Inc (US), Dow Agrosciences Llc (US) (2006)	US	Group of plants (including algae and trees)	Targeted mutation: changed composition	ZFN 1,2
Carroll, D., Bibikova, M., Drews, G. N., Golic, K. G. & Golic, M. M. Targeted chromosomal mutagenesis using zinc finger nucleases. WO/2003/087341. University of Utah Research Foundation (US), (2002).	US	Crop plants + Arabidopsis	Targeted insertion: multiple traits	ZFN 3
Dekelver, R., Holmes, M. C., Urnov, F. & Gregory, P. D. Linear Donor Constructs For Targeted Integration, WO/2009/131632. Sangamo Biosciences Inc (US) (2008)	US	Plants in general	Targeted insertion: multiple traits	ZFN 3
Dekelver, R., Gupta, M., Miller, J. C., Novak, S. & Petolino, J. F. Engineered zinc finger proteins targeting plant genes involved in fatty acid biosynthesis. WO/2011/049627. Dow Agrosciences LLC (US), Sangamo Biosciences Inc (US), (2009).	US	Brassica plants	Targeted insertion: modified fatty acid composition	ZFN 3
Doyon, Y. Organisms homozygous for targeted modification. WO/2011/019385. Sangamo Biosciences Inc (US) (2009).	US	group of plants (Arabidopsis + crop plants)	Targeted insertion	ZFN 3
Gupta, M., Palta, A., Novak, S., Urnov, F. & Gopalan, S. Engineered Zinc Finger Proteins Targeting 5-Enolpyruvyl Shikimate-3-Phosphate Synthase Genes, WO/2009/042164. Dow Agrosciences Llc (US), Sangamo Biosciences Inc (US) (2007)	US	Plants in general	Targeted mutation: herbicide tolerance	ZFN 1,2
Hlubek, A., Biesgen, C. & Hoeffken, H. W. Chimeric endonucleases and uses thereof. WO/2011/064750. BASF China LTD (CN), BASF Plant Science Co GMBH (DE), (2009).	CN, DE	Plants in general	Targeted mutation	ZFN 1,2
Kausch, A. P. & Dellaporta, S. Male and female sterility lines used to make hybrids in genetically modified plants. WO/2011/090752. Rhode Island Board of Governors for Higher Education (US) (2009).	US	Plants in general	Targeted mutation: male/female sterility	ZFN 1
Kim, J. S. & Kim, H. J. A novel zinc finger nuclease and uses thereof. WO/2010/076939. Toolgen (KR) (2008).	KR	Plants in general	Targeted mutation and insertion	ZFN 1,2,3
Kim, J. S., Lee, H. J. & Kim, E. J. Targeted genomic rearrangements using site-specific nucleases. WO/2010/143917. Toolgen (KR) (2009).	KR	Plants in general	Targeted mutation and insertion	ZFN 1,2,3
Liljedahl, M., Aspland, S. E. & Segal, D. J. Methods And Compositions For Using Zinc Finger Endonucleases To Enhance Homologous Recombination, WO/03/080809. Sangamo Bioscience Inc (US) (2002)	US	Plants in general	Targeted insertion: multiple traits	ZFN 3
Miller, J. C. Variant foki cleavage half-domains. EP/2213731. Sangamo Biosciences Inc (US) (2006).	US	Plants in general	Targeted mutation	ZFN 2

Miller, J. et al. Zinc finger nuclease-mediated homologous recombination, WO/2008/021207 Sangamo Biosciences Inc (US), Dow Agrosciences LLC (US), (2006).	US	Plants in general	Targeted insertion: a protein, a marker gene or a siRNA	ZFN 3
Miller, J. C. Engineered Cleavage Half-Domains, US/2009/311787. Sangamo Biosciences Inc (US) (2006)	US	Plants in general	Targeted mutation	ZFN 2
Miller, J. C. Compositions For Linking Dna-Binding Domains And Cleavage Domains, WO/2009/154686. Sangamo Biosciences Inc (US) (2008)	US	Plants in general	Targeted mutation	ZFN 1,2
Miller, J. C. & Zhang, L. Methods And Compostions For Targeted Cleavage And Recombination, WO/2005/084190. Sangamo Biosciences Inc (US) (2004)	US	Plants in general	Sequence replacement	ZFN 3
Petolino, J., Cai, C. & Ni, W. Protein Production In Plant Cells And Associated Methods And Compositions, WO/2010/019386. Dow Agrosciences Llc (US), Sangamo Biosciences Inc (US) (2008)	US	Tabacco + group of dicots + group of monocots	Targeted insertion: protein production	ZFN 3
Rolland A., Dubald M., Van Lookeren-Campagne M. & Ruiter R. Methods And Means For Exact Replacement Of Target Dna In Eukaryotic Organisms, WO/2008/148559. Bayer Bioscience Nv (BE), Bayer Cropscience Sa (FR) (2007)	BE,FR	Plants in general	Sequence replacement	ZFN 3
Russel, S. & Petolino, J. F. Excision of transgenes in genetically modified organisms. WO/2011/091311. Dow Agrosciences LLC (US) (2010).	US	Plants in general	Excision of a DNA sequence	
Samuel, J., Petolino, J., Samboju, N., Webb, S. & Yau, K. Nanoparticle mediated delivery of sequence specific nucleases. WO/2010/118077. Dow Agrosciences LLC (US) (2009).	US	Arabidopsis + crop plants	introduction of ZFN into plant cells through nanoparticles	
Sriram, S., Elango, N., Sastry-Dent, L. & Petolino, J. Data analysis of DNA sequences. WO/2012/092039. Dow Agrosciences LLC (US) (2010).	US	Plants in general	Targeted mutation	ZFN 1
Toki, S. & Osakabe, K. Production method for genetically modified plant cells. WO/2011/052539. National Institute of Agrobiological Sciences (NIAS) (JP) (2009).	JP	Plants in general	Targeted mutation	ZFN 1
Vainstein, A. & Zuker, A. Plant Viral Expression Vectors And Use Of Same For Generating Genotypic Variations In Plant Genomes, WO/2009/130695. Danziger Innovation Ltd (IL) (2008)	IL	Petunia, Tabaco, Arabidopsis + other plants	Targeted insertion: male sterilty Targeted mutation: herbicide tolerance	ZFN 1,2,3
Vainstein, A. & Zuker, A. Generating genotypic variations in plant genomes by gamete infection. WO/2011/048600. Danziger Innovations LTD (IL) (2009).	IL	Petunia, Tabaco, Arabidopsis + other plants	Targeted mutation: male sterility + herbicide tolerance	ZFN 1
Wang, J. Methods And Compositions For Targeted Single-Stranded Cleavage And Targeted Integration, WO/2010/021692. Sangamo Biosciences Inc (US) (2008)	US	Plants in general	Sequence replacement	ZFN 3

Meganuclease (MGN) techniques (MGN1,2,3)

Patent	Country	Plant	Trait	MGN 1,2,3
Arnould, S. et al. Custom-made meganuclease and use thereof. WO/2004/067736. Cellectis (FR), (2003).	FR	Plants in general	Targeted mutation	MGN1
Arnould, S., Chames, P., Choulika, A., Epinat, J. C. & Lacroix, E. Hybrid and single chain meganucleases and use thereof. WO/03/078619. Cellectis (FR), (2002).	FR	Plants in general	Targeted mutation and insertion	MGN1,3
Choulika, A., Perrin, A., Dujon, B. & Nicolas, J. F. Nucleotide sequence encoding enzyme I-SceI and use thereof. JP/2007/014347. Pasteur Institute (FR), University Pierre and Marie Curie (FR), (1994).	FR	Plants in general	Targeted insertion	MGN3

D'Halluin, K. Improved plant transformation methods. WO/2006/074956. Bayer Biosciences NV (BE), (2005).	BE	Maize, Tobacco + group of plants	Targeted insertion	MGN3
D'Halluin, K. Methods and means to modify a plant genome at a nucleotide sequence commonly used in plant genome engineering. WO/2011/154159. Bayer Bioscience NV (BE), (2011).	BE	Plants in general	HT, biotic and abiotic resistance, modified composition	MGN3
D'Halluin, K. & Ruiter, R. Methods and means for removal of a selected DNA sequence. WO/2008/037436. Bayer Bioscience NV (BE), (2006).	BE	Plants in general	Removal and substitution of DNA sequence	
D'Halluin, K., Van Der Straeten, C. & Ruiter, R. Improved targeted DNA insertion in plants. WO/2005/049842. Bayer Biosciences NV (BE), (2003).	BE	Maize	Targeted insertion	MGN3
Duchateau, P., Juillerat, A., Silva, G. H. & Epinat, J. C. Method for increasing the efficiency of double-strand break-induced mutagenesis. WO/2012/058458. Cellectis (FR), (2010).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Duchateau, P. & Paques, F. Heterodimeric meganucleases and use thereof. WO/2007/034262. Cellectis (FR), (2005).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Epinat, J. C. & Lacroix, E. I-Dmoi Derivatives with Enhanced Activity at 37oC and Use Thereof. US/2008/271166. Cellectis (FR), (2004).	FR	Plants in general	Targeted mutation	MGN1
Fajardo Sanchez, E. et al. Obligate heterodimer meganucleases and uses thereof. EP/2433641. Cellectis (FR), (2007).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Gordon-Kamm, W. L. et al. Methods and compositions for targeted polynucleotide modification. WO/2011/082310. Pioneer Hi Bred Int (US), (2009).	US	Maize + group of monocots	Targeted insertion	MGN3
Grizot, S. I-MsoI homing endonuclease variants having novel substrate specificity and use thereof. WO/2009/068937. Cellectis (FR), (2007).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Grizot, S. New I-CreI derived single-chain meganuclease and uses thereof. WO/2009/095793. Cellectis (FR), (2008).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Grizot, S. & Duchateau, P. Chimeric meganuclease enzymes and uses thereof. WO/2009/074873. Cellectis (FR), (2007).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Grizot, S. & Duchateau, P. Improved chimeric meganuclease enzymes and uses thereof. WO/2009/074842. Cellectis (FR), (2007).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Grizot, S. & Gouble, A. Method for enhancing the cleavage activity of I-CreI derived meganucleases. WO/2009/001159. Cellectis (FR), (2007).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Hlubek, A. & Biesgen, C. Optimized endonucleases and uses thereof. WO/2011/064736 BASF China Co Ltd (CN), BASF Plant Science Co GMBH (DE), (2009).	CN, DE	Plants in general	Targeted mutation	MGN1
Jantz, D. & Smith, J. J. Rationally-designed meganucleases for maize genome engineering. WO/2009/114321 Precision Biosciences (US), (2008).	US	Maize	Targeted insertion	MGN3
Jantz, D. & Smith, J. J. Recognition sequences for I-CreI-derived meganucleases and uses thereof. WO/2010/009147. Precision Biosciences (US), (2008).	US	Plants in general	Targeted mutation	MGN1
Lyznik, L. A., Tao, Y. & Gao, H. Methods for altering the genome of a monocot plant cell. EP/2167666. Pioneer Hi Bred Int (US), (2007).	US	Maize + group of monocots	Targeted mutation and insertion	MGN1,2,3
Marcaide Lopez, M. J., Prieto Lugo, F. J. & Montoya Blanco, G. The crystal structure of I-DmoI in complex with its DNA target, improved chimeric meganucleases and uses thereof. WO/2010/001189. Cellectis (FR), (2008).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Montoya, G., Blanco, F. & Prieto, J. LAGLIDADG homing endonuclease variants having novel substrate specificity and uses thereof. WO/2008/102198. Cellectis (FR), (2007).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Paques, F. I-CreI homing endonuclease variants having novel cleavage specificity and use thereof. WO/2007/060495. Cellectis (FR), (2005).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3

Paques, F. I-CreI meganuclease variants with modified specificity, method of preparation and uses thereof. WO/2006/097784. Cellectis (FR), (2005).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Paques, F. LAGLIDADG homing endonuclease variants having mutations in two functional subdomains and use thereof. WO/2007/057781. Cellectis (FR), (2005).	FR	Plants in general	Targeted insertion	MGN3
Paques, F., Grizot, S. & Duchateau, P. Novel method to generate meganucleases with altered characteristics. WO/2010/015899. Cellectis (FR), (2008).	FR	Plants in general	Targeted mutation and insertion	MGN1,2,3
Paul, W., Wehrkamp-Richter, S. & Laffaire, J. B. Method for performing homologous recombination in plants. WO/2007/135022. Biogemma (FR), (2006).	FR	Plants in general	Targeted mutation	MGN1
Rougeon, F., Azzouz-Boubakour, I., Lopez, B. & Bertrand, P. Use of terminal deoxynucleotidyl transferase for mutagenic DNA repair to generate variability, at a determined position in DNA. EP/2412806. Pasteur Institute (FR), Commissariat a l'Energie Atomique et aux Energies Alternatives (FR), (2010).	FR	Plants in general	Targeted mutation	MGN1

Transcription Activator-Like Effector Nuclease (TALEN) techniques

Patent	**Country**	**Plant**	**Trait**	**TALEN 1,2,3**
Boch, J., Bonas, U., Lahaye, T. & Schornack, S. Modular DNA-binding domains and methods of use. WO/2010/079430. (DE) (GB) (2009).	DE, GB	Plants in general	sequence recognition and binding	
Kim, J. S. & Kim, H. J. Genome enginerring via designed TAL effector nucleases. WO/2012/093833. Toolgen Inc (KR), Seoul National University R&DB Foundation (KR), (2011).	KR	Plants in general	Targeted mutation and insertion	TALEN 1,2,3
Kuehn, R., Wurst, W. & Meyer, M. Fusion proteins comprising a DNA-binding domain of a TAL effector protein and a non-specific cleavage domain of a restriction nuclease and their use. WO/2011/154393. Helmholtz Zentrum Muenchen (DE), (2010).	DE	Plants in general	Targeted mutation and insertion	TALEN 2,3
Voytas, D. F. et al. TAL effector-mediated DNA modification. WO/2011/072246. University of Minnesota (US), University of Iowa Research Foundation (US), (2009).	US	Plants in general	Targeted mutation	TALEN 1
Yang, B., Li, T. & Huang, S. Nuclease activity of TAL effector and FokI fusion protein. WO/2011/159369. University of Iowa Research Foundation (US), (2010).	US	Plants in general	Targeted mutation	TALEN 1

RNA-dependent DNA methylation (RdDM)

Patent	**Country**	**Plant**	**Trait**
Wassenegger, M., Krczal, G. & Dalakouras, A. Method For The Production Of A Transgene-Free Plant With Altered Methylation Pattern, WO/2010/066343. Rlp Agroscience Gmbh (DE) (2008)	DE	Plants in general	Silencing of a harmful gene or an unwanted trait

Reverse Breeding

Patent	Country	Plant	Trait
Dirks, R. H. G., Van Dun, C. M. P. & Reinink, K. Reverse Breeding, WO/03/017753. Rijk Zwaan Zaadteelt En Zaadha (NL) (2001)	NL	Plants in general	Creation of parental lines for the production of F1 hybrid seed.
Van Dun, C. M. P. & Dirks, R. H. G. Near Reverse Breeding, WO/2006/094773. Rijk Zwaan Zaadteelt En Zaadha (NL) (2005)	NL	Plants in general	Creation of parental lines for the production of F1 hybrid seed.

Cisgenesis and Intragenesis

Patent	Country	Plant	Trait
Allefs, J. J. H. M. & Van Der Vossen, E. A. G. Gene Conferring Resistance To Phytophthora Infestans (Late-Blight) In Solanacea, WO/03/066675. Kweek En Researchbed Agrico Bv (NL) (2002)	NL	Solanaceae	Fungal resistance
Conner, A. *Et Al.* Plant Transformation Using Dna Minicircles, WO/2010/090536. New Zealand Inst For Plant And (NZ) (2009)	NZ	Plants in general	Intragenic transformation
Conner, A. J. *Et Al.* Transformation Vectors, WO/2005/121346. The New Zealand Institute For Plant And Food Research Limited (NZ) (2004)	NZ	Plants in general	Intragenic transformation
De Vetten, N. C. M. H., Visser, R. G. F., Jacobsen, E., Van Der Vossen, E. A. G. & Wolters, A. M. A. Use Of R-Genes As A Selection Marker In Plant Transformation And Use Of Cisgenes In Plant Transformation, WO/2008/091154. Cooeperatie Avebe U A (NL) (2007)	NL	Solanaceae	Fungal resistance
Halterman, D. & Liu, Z. Late Blight Resistance Gene From Wild Potato, WO/2009/023755 Wisconsin Alumni Res Found (US) (2007)	US	Potato, tomato, tobacco	Fungal resistance
Jacobsen, E., Visser, R. G. F., Van Der Vossen, E. A. G. & Vleeshouer, V. Identification, Classification And Optionally Stacking Of R-Genes In Solanum Using An Effector-Receptor Approach, EP/1950304. Cooeperatie Avebe U A (NL) (2007)	NL	Solanum plants	Fungal resistance
Jahn, M. & Cavatorta, J. Mutated EIF4E sequences from potato which are useful in imparting virus resistance. WO/2010/048398. Cornell University (US), (2008).	US	Potato	Virus resistance
Jones, J. *Et Al.* Late Blight Resistance Genes And Methods, WO/2009/013468. Wageningen University (NL), Plant Bioscience Ltd (GB) (2007)	NL, GB	Potato	Fungal resistance
Luo, J., Butelli, E., Jones, J., Tomlinson, L. & Martin, C. R. Methods And Compositions For Modifying Plant Flavonoid Composition And Disease Resistance, WO/2009/103960. Norfolk Plant Sciences Ltd (GB) (2008)	GB	Solanaceae (potato, tomato)	Changed composition, Fungal resistance
Osumi, T., Belknap, W. R., Rockhold, D. R. & Maccree, M. M. Solanum Bulbocastanum Late Blight Resistance Gene And Use Thereof, WO/2004/020594. Us Agriculture (US) (2002)	US	Potato	Fungal resistance
Richael, C. Generation of marker-free and backbone-free transgenic plants using a single binary approach. WO/2007/092308. Simplot Co J R (US), (2006).	US	Dicots	Lower acrylamide level, Reduced black- spot bruising, Reduced cold-induced sweetening
Rommens, C. Plant-Specific Genetic Elements And Transfer Cassettes For Plant Transformation, WO/2008/082429. Simplot Co J R (US) (2004)	US	Group of monocots + group of dicots	Intragenic transformation
Rommens, C. Low Acrylamide Foods, WO/2007/035752. Simplot Co J R (US) (2005)	US	Potato	Lower acrylamide level

Rommens, C., Yan, H. & Ye, J. Reduced Acrylamide Plants And Foods, US/2009/123626. Simplot Co J R (US) (2007)	US	Potato + group of crop plants	Lower acrylamide level
Rommens, C. M. T. *Et Al.* Precise Breeding, WO/03/069980. Simplot Co J R (US) (2002)	US	Potato, wheat + group of dicots + group of monocots	Reduced black-spot bruising, Reduced cold-induced sweetening + other traits
Rommens, C. M., Duan, H. & Chawla, R. Potyvirus resistance in potato. WO/2012/054468. Simplot Co J R (US), (2010).	US	Potato	Virus resistance
Van Der Vossen, E. A. G., Lokossou, A. A., Visser, R. G. F. & Jacobsen, E. A Functional R-Gene From Solanum Bulbocastanum, WO/2008/091153. Wageningen Universiteit (NL), Kweek-en Researchbedrijf Agrico B.V. (NL) (2007)	NL	Potato	Fungal resistance
Van Der Vossen, E. A. G., Allefs, J. J. H. & Muskens, M. W. M. Fungus resistant plants and their uses. WO/2005/014631. Kweek-en Researchbedrijf Agrico B.V. (NL), (2003).	NL	Potato	Fungal resistance
Van Der Vossen, E. A. G., Van Der Voort, J. N., Lankhorst, R. M. K., Bakker, J. & Stiekema, W. J. Engineering Nematode Resistance In Solanacae, WO/0006754. Wageningen University (NL) (1998)	NL	Potato	Resistance vs nematodes
Weeks, T. J. & Rommens, C. M. T. Refined Plant Transformation, WO/03/079765. Simplot Co J R (US) (2003)	US	Group of monocots + group of dicots	Intragenic transformation: group of traits

Grafting non-GM varieties onto GM rootstocks

Patent	Country	Plant	Trait
Aldwinckle, H. S. & Norelli, J. L. Transgenic Pomaceous Fruit With Fire Blight Resistance, WO/94/07356 Cornell Res Foundation Inc (US) (1992)	US	Apple, pear	Resistance vs bacteria
Allen, E. *Et Al.* Invertebrate Micrornas, WO/2008/103643. Monsanto Technology Llc (US) (2007)	US	Maize, grape, apple, soybean	Resistance vs insects and nematodes
Czosnek, H. Virus Tolerant Plants And Methods Of Producing Same, WO/2008/102337. Yissum Res Dev Co (IL) (2007)	IL	Crop plants	Virus resistance
Fengwang, M. A., Yonghong, L., Fengjuan, F., Shouguo, S. & Yanzi, Z. Method for culturing apple seedling by agrobacterium-mediated high-efficiency transformation system. CN/102031270. Northwest A&F University (CN), (2009).	CN	Apple	Salt resistance
Gal-On, A., Zelcer, A., Wolf, D., Gaba, V. P. & Antignus, Y. Engrafted Plants Resistant To Viral Diseases And Methods Of Producing Same, WO/2005/079162 Israel State (IL) (2004)	IL	Cucumber	Virus resistance
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Polston, J. E. & Hiebert, E. Materials And Methods For Providing Resistance To Plant Pathogens In Non-Transgenic Plant Tissue, WO/2005/118805. Univ Florida (US) (2004)	US	Tomato + group of dicots	Resistance to virus + group of pathogens
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Schnabel, G., Scorza, R. & Layne, D. Increased Resistance Of Plants To Pathogens From Multiple Higher-Order Phylogenetic Lineages. Clemson University Research Foundation (US) (2006)	US	Edible crop plants	Fungal resistance
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Zhu, H., Ling, K. & Gonsalves, D. Grapevine Leafroll Virus (Type 2) Proteins And Their Uses, WO/98/53055. Cornell Res Foundation Inc (US) (1997)	US	Grapevine, citrus, beet, tobacco	Virus resistance