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Maria Lusser, Claudia Parisi, Damien Plan, Emilio Rodríguez-Cerezo

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European Commission
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Contact information
Address: Edificio Expo. • c/ Inca Garcilaso 3 • E-41092 Seville (Spain)
E-mail: jrc-ipts-secretariat@ec.europa.eu
Tel.: +34 954488318
Fax: +34 954488300

http://ipts.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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NEW PLANT BREEDING TECHNIQUES
STATE-OF-THE-ART AND PROSPECTS FOR COMMERCIAL DEVELOPMENT

Authors:
Maria Lusser[1], Claudia Parisi[1], Damien Plan[2], Emilio Rodríguez-Cerezo[1]

[1] European Commission, Joint Research Centre (JRC), Institute for Prospective Technological Studies (IPTS)

[2] European Commission, Joint Research Centre (JRC), Institute for Health and Consumer Protection (IHCP)

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Katia Pauwels	Scientific Institute of Public Health (IPH), BE
Jaroslava Ovesna	Crop Research Institute (VURV), CZ
Theo W. Prins	Institute of Food Safety (RIKILT), NL
Slawomir Sowa	Plant Breeding and Acclimatisation Institute (IHAR), PL
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Jan G. Schaart	Wageningen UR, Plant Breeding, NL
Anne-Marie Wolters	Wageningen UR, Plant Breeding, NL

PREFACE

The study "New Plant Breeding Techniques: State-of-the-Art and Prospects for Commercial Development" was carried out in 2010, responding to an initial request from the Directorate-General for the Environment (DG ENV) of the European Commission, to provide information on the state of adoption and possible economic impact of new plant breeding techniques. From February 2010, the DDirectorate-General for Health and Consumers (DG SANCO) became responsible for relevant legislation on biotechnology (Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms[1]) and therefore the main customer of this study.

The study was developed and led by the European Commission's Joint Research Centre (JRC) Institute for Prospective Technological Studies (IPTS) in cooperation with the JRC Institute for Health and Consumer Protection (IHCP).

Among other sources, the report draws on information from a workshop organised on 27-28 May 2010 in Seville and a survey directed at plant breeding companies. Evaluations of specific aspects of new plant breeding techniques (evaluation of changes in the plant genome and evaluation of possibilities for detection) were carried out by two working groups of external experts coordinated by the JRC-IPTS and JRC-IHCP, respectively.

1 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 - OJ L 106, 17.4.2001, p. 1–39

EXECUTIVE SUMMARY

Background

Innovation in plant breeding is necessary to meet the challenges of global changes such as population growth and climate change. Agriculture has been able to cope with these challenges until now. However, further efforts are needed and therefore plant breeders search for new plant breeding techniques.

Harmonised EU legislation regulating genetically modified organisms (GMOs) goes back to the year 1990. The GMO legislation has been revised between 2001 and 2003. However, the definition of GMOs remains the same as in 1990. Plant breeding techniques which have been developed over the last 10 years therefore create new challenges for regulators when applying the GMO definition.

A working group established by the European Commission in 2007 is currently evaluating whether certain new techniques constitute techniques of genetic modification and, if so, whether the resulting organisms fall within the scope of the EU GMO legislation. The group is discussing the following eight new techniques[2]:

- Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
- Oligonucleotide directed mutagenesis (ODM)
- Cisgenesis and intragenesis
- RNA-dependent DNA methylation (RdDM)
- Grafting (on GM rootstock)
- Reverse breeding
- Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)
- Synthetic genomics

2 Short definitions of the techniques are listed in Annex 9.

This Study

This study focuses on the same list of techniques[3]. It investigates the degree of development and adoption by the commercial breeding sector of new plant breeding techniques and discusses drivers and constraints for further development of new plant varieties based on these techniques. It also reviews knowledge of the changes in the genome of plants induced by these techniques and highlights studies on food, feed and environmental safety. Finally the study evaluates the technical possibilities for detecting and identifying crops produced by new plant breeding techniques.

Research And Development

A scientific literature search was performed in order to evaluate the development of research activities. A total of 187 publications were identified. The results of the research show that the new plant breeding techniques discussed in this report are still young. Publication started only 10 years ago, with the exception of grafting on GM rootstock (20 years).

Concerning the geographical distribution of publications, the EU is leading (with 45% of all publications) followed by North America (32%). The majority of publications (81%) are produced by public institutes, followed by collaborations between public and private institutes and private companies.

Patenting Activities

In a patent search, a total of 84 patents related to new plant breeding techniques were identified, most of which were filed during the last decade (showing similar development in time as publications). The majority of patent applications comes from applicants based in the USA (65%), followed by EU-based applicants (26%).

3 No research relevant to the use of synthetic genomics in plant breeding is under way or is likely to be undertaken in the near future. Therefore, no literature or patent search was carried out, nor was synthetic genomics included in the survey directed at companies applying biotechnology to plant breeding, nor were the changes in the genome or detection issues discussed for synthetic genomics.

The majority of patent applications were from private companies (70%), followed by universities/ public research institutions (26%) and private/ public collaborations (4%). Patenting shows the high specialisation of the 50 companies/institutes active in the field. Most of them old patents for only one of the techniques.

Commercial Pipeline

To ascertain to what extent the new plant breeding techniques have already been adopted by the plant breeding sector a survey of plant breeding companies was carried out. The information was complemented with data obtained during a workshop with participants from the public and private sectors and a search in a database of applications for field trials in the EU.

The results of the survey show that all of the seven new plant breeding techniques have been adopted by commercial breeders. ODM, Cisgenesis and agro-infiltration are the most used techniques and the crops developed with these techniques have already commercial development phase I-III[4]. ZFN technique, RdDM, grafting on GM rootstocks and reverse breeding are less used techniques and are still mainly applied at research level. It is estimated that the most advanced crops are close (2-3 years) to commercialisation (in the event of the techniques being classified as non-GM techniques).

Drivers For Adoption

The main driver for the adoption of new plant breeding techniques is the great technical potential of these techniques. Some of them allow targeted mutagenesis or the site specific introduction of new genes, others result in the silencing of genes. For many of the techniques the genetic information coding for the desired trait is only transiently present in the plants or stably integrated only in intermediate plants. Therefore, the commercialised crop will not contain an inserted transgene[5].

The second main driver for the adoption of new plant breeding techniques is its economic advantages. The use of new plant breeding techniques makes the breeding process faster which lowers the production costs. For example, cisgenesis uses the same gene pool as conventional cross breeding, but is much faster as it avoids many steps of back-crossing.

Constraints For Adoption

The main technical constraints on the development and adoption of new plant breeding techniques concern the efficiency, which is currently generally low for many of the techniques. Furthermore, the methods for delivering the genetic information into the plant cell, for the regeneration of plants and from cuttings and methods for successfully altered plants have to be further developed.

The regulatory costs for GM crops are very high. Therefore, the legal status of the new plant breeding techniques will influence the decision on whether to use these techniques only for the introduction or modification of traits in crops with very high value or more extensively for a broad field of applications.

4 PHASE I: Gene optimisation, crop transformation
PHASE II: Trait development, pre-regulatory data, large-scale transformation
PHASE III: Trait integration, field testing, regulatory data generation (if applicable)
PHASE IV: Regulatory submission (if applicable), seed bulk-up, pre-marketing

5 For a definition of transgenesis see Annex 9. Transgenesis is a technique of genetic modification (Directive 2001/18/EC, Annex 1A, Part 1 (1)).

Challenges For Detection

Availability of detection methods is a regulatory requirement for GMOs under the EU legislation. Therefore the possibilities for detecting and identifying crops produced with new plant breeding techniques were investigated by an ad hoc task force of experts. The task force concluded that DNA is the best target molecule for unambiguously detecting and identifying a change in the genetic material of plants, and that amplification-based methods (polymerase chain reaction, PCR) are the most appropriate for this purpose.

When the resulting genetic modification cannot be distinguished from those produced by conventional breeding techniques or by natural genetic variation, it is not possible to develop detection methods that provide unambiguous results. The task force concluded that identification of genetic modification is currently not possible for the following techniques: ZFN-1 and -2, ODM, RdDM, grafting on GM rootstock, reverse breeding, agro-infiltration "sensu stricto" and agro-inoculation. Only for the following techniques, which lead to insertions of new genes, is identification possible, provided information about the DNA sequence introduced and the neighbouring sequence is available: ZFN-3 technology, cisgenesis/intragenesis and floral dip.

Abbreviations

1-D/2-D	one/two dimensional
AHAS	Acetohydroxyacid Synthase
ALS	Acetolactate Synthase
BAC	Biosafety Advisory Council
bp, kbp	base pairs, kilo base pairs
CA	Competent Authority
CEN	European Committee for Standardisation
C-LEC1	Carrot-Leafy Cotyledon 1
COGEM	Dutch Commission on Genetic Modification
DAS	Double Antibody Sandwich
ddNTPs	dideoxynucleotides triphosphates
DG ENV	Directorate-General for the Environment
DG SANCO	Directorate-General for Health and Consumers
DH	Doubled Haploid
DiGE	Difference Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
dsRNA	double stranded RNAs
EC	European Commission
ECLA	European Classification
EI	Electron Ionisation
ELISA	Enzyme Linked Immuno Sorbent Assay
EMS	Ethyl Methane Sulfonate
ENGL	European Network of GMO Laboratories
EPO	European Patent Office
ESI	Electro Spray Ionisation
EU	European Union
EU-RL GMFF	European Union Reference Laboratory for GM Food and Feed
FAO	Food and Agriculture Organization of the United Nations
FT-MS	Fourier Transform Mass Spectrometry
GBSS	Granule-Bound Starch Synthase
GC	Gas Chromatography
GFP	Green Fluorescent Protein
GM	Genetically Modified
GMM	Genetically Modified Micro-organism
GMO	Genetically Modified Organism
GUS	Beta-glucuronidase gene
GVA	Grapevine virus A
HILIC	Hydrophilic Interaction Chromatography
HPLC	High performance Liquid Chromatography
hpRNA	hairpin RNA
HR	Homologous Recombination
HRM	High-Resolution Melting
IHCP	Institute for Health and Consumer Protection
IPTS	Institute for Prospective Technological Studies
ISO	International Organisation for Standardisation
JRC	Joint Research Centre
LC	Liquid Chromatography
LFD	Lateral Flow Devices
LNA	Locked Nucleic Acids
LOD	Limit Of Detection
LOQ	Limit Of Quantification

MALDI	Matrix-Assisted Laser-Desorption Ionisation
MAS	Marker Assisted Selection
miRNA	micro RNA
mRNA	messenger RNA
MS	Member States
MS	Mass Spectrometry
MS-HRM	Methylation-Sensitive High-Resolution Melting
ncRNA	non-coding RNA
NHEJ	Non-Homologous End-Joining
NMR	Nuclear Magnetic Resonance
NOS	Nopaline Synthase
NPTII	Neomycin Phosphotransferase Gene
nt	nucleotides
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 Frames
PAGE	Polyacrylamide Gel Electrophoresis
PAT	Phosphinothricin phosphotransferase
PCR	Polymerase Chain Reaction
PCT	Patent Cooperation Treaty
PEG	Polyethylene Glycol
PTA	Plate Trapped Antigen
PTGS	Post-Transcriptional Gene Silencing
R&D	Research and Development
RdDM	RNA-dependent DNA Methylation
RIKILT	Institute of Food Safety of Wageningen University
RNA	Ribonucleic Acid
RNAi	RNA interefrence
RP	Reversed-Phase
rRNA	ribosomal RNA
RT qPCR	Real-Time quantitative PCR
siRNA	small interfering RNA
SNPs	Single Nucleotide Polymorphisms
TAS	Triple Antibody Sandwich
T-DNA	Transfer DNA
TFO	Triple helix-Forming Oligonucleotide
TGS	Transcriptional Gene Silencing
TOF	Time Of Flight
tRNA	transfer RNA
UHPLC	Ultra High Performance Liquid Chromatography
USPTO	United States Patent and Trademark Office
UV	Ultra-Violet
WIPO	World Intellectual Property Organization
ZFN	Zinc Finger Nuclease

Table of Contents

1. INTRODUCTION

Innovation in plant breeding is necessary to meet the challenges of global changes such as population growth and climate change. Because of the increase in world population and the need to protect the environment, the limited resources of land and water have to be used more efficiently for crop production. On the basis of statistics from the FAO, food production must be doubled between 2000 and 2050. Additionally, consumers demand healthy food and high value ingredients. Therefore, plants with useful traits for pest resistance, disease, herbicide and stress tolerance and improved product quality characteristics have to be developed.

Agriculture has been able to cope with these challenges until now. A considerable yield increase has been achieved for many crops, e.g. 120 kg/ha/year for corn within the last 20 years. In addition to a more efficient land, energy and water use, soil loss and greenhouse gas emissions per unit of agricultural output have been reduced during recent years by the use of improved varieties and agricultural techniques. Further efforts are however needed and therefore plant breeders search for new plant breeding techniques as an additional tool to meet these objectives.

Plant breeding started 10 000 years ago by selecting the best plants in the field, leading to domestication. The discovery of the law of genetics by Gregor Mendel about 150 years ago enhanced the speed of plant breeding considerably. The invention of cross breeding was followed by hybrid breeding in the 1930s, tissue and cell culture methods in the 1960s and recombinant DNA techniques and genetic engineering in the 1980s. So-called "smart breeding" started in the late 1990s with the use of molecular markers, genome mapping and sequencing.

The development of new techniques in plant breeding did not lead to the replacement of the older methods. The use of all available technologies is essential for plant breeding. Conventional breeding techniques, transgenesis and new plant breeding techniques are essential components of what we could call the plant breeders' toolbox.

Harmonised EU legislation regulating organisms produced by modern bio-techniques (genetically modified organisms, GMOs) dates back to the year 1990[6]. The GMO legislation has been revised during recent years and additional legislation was introduced in 2003 to regulate food and feed derived from GMO crops. However, the definition of GMOs remains the same as in 1990. Therefore, it does not reflect the state-of-the-art of modern breeding technologies.

During the last 20 years new biotechnological techniques and especially new plant breeding techniques have been developed. They create new challenges for regulators when applying the GMO definition from 1990. Crops produced using some of these new plant breeding techniques cannot be distinguished from their conventionally bred counterparts and therefore there are claims that they should be exempted from the GMO legislation.

Regulatory costs for plant varieties classified as GMOs are much higher than those needed for the registration and approval of non-GM plant varieties. Biotechnology companies and plant breeders, especially small and medium businesses, are particularly concerned about the legislative uncertainty of the GMO classification.

At the request of Competent Authorities (CAs) of EU Member States, a working group was established by the European Commission (EC) in October 2007 to evaluate a list of eight new techniques proposed by the CAs. The objective of this "New Techniques Working Group" (NTWG) is to examine new techniques in the context of GMO legislation. The NTWG is currently analysing whether these techniques constitute techniques of genetic modification and, if so, whether the resulting organisms fall within the scope of the EU GMO legislation.

The study presented here evaluates the same list of plant breeding techniques. However, the focus is on the status of development of research on these techniques and the degree of adoption by the breeding sector, their potential development of commercial products and the challenges for detecting products derived from these techniques.

6 For further information on the EU GMO legislation, the revision and current evaluation refer to Annex 1, Legal Background. For further information on the EU definition of GMOs refer to Chapter 3 and Annex 2, GMO Definition.

2. THIS STUDY

The study forms part of the activities of the Institute for Prospective Technological Studies (IPTS) and the Institute for Health and Consumer Protection (IHCP), two of the institutes of the European Commission's Joint Research Centre (JRC).

The overall objective of the study is to identify the degree to which new plant breeding techniques are developed and adopted by the breeding sector and the potential of the techniques for breeding commercial crop varieties. It addresses the state-of-the-art of research and development in the EU, as well as in non-EU countries, especially the USA and Japan. It evaluates the changes in the genome of plants, highlights studies on environmental and consumer risk issues and discusses drivers and constraints for further commercial adoption of these technologies. Finally, the study provides an evaluation of the difficulties of detecting crops produced by the new plant breeding techniques.

The study focuses on the following eight new plant breeding techniques[7]:

- Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
- Oligonucleotide directed mutagenesis (ODM)
- Cisgenesis and intragenesis
- RNA-dependent DNA methylation (RdDM)
- Grafting (on GM rootstock)
- Reverse breeding
- Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)
- Synthetic genomics[8]

The report is structured as follows. Chapter 3 provides definitions of the technologies studied, beginning with the GMO definition under the EU legislation and followed by definitions for each of the new plant breeding techniques.

Chapter 4 presents the state-of-the-art of research and patenting activities including a comprehensive analysis of the actors involved. It also includes an analysis of the current adoption of these technologies by the breeding industry and the prospects for a pipeline of commercial development of crops based on these technologies. The chapter draws on information obtained from literature and a patent search and from a workshop, a survey of breeding companies and a search in a database of notifications of field trials.

Drivers and constraints for the adoption of the new plant breeding techniques are discussed in Chapter 5. Information on the technical and economical advantages of the new technologies compared to current practices and on the constraints and challenges for adoption comes from the literature search, the survey, discussions with experts at Wageningen UR, Plant Breeding, NL and the workshop.

Chapter 6 evaluates changes in the plant genome caused by the application of the new plant breeding techniques.

Chapter 7 deals with issues related to detecting and identifying crops resulting from the application of the new plant breeding techniques. This chapter draws on the work of an ad hoc task force of experts.

Further needs for technical research and new breeding techniques, not included in this project but identified during the course of our research, are presented in Chapter 8.

7 Note: The term "new plant breeding techniques" refers to the mandate given to the JRC. This does not necessarily mean that those techniques have not been applied before either in plant breeding or other biotechnological applications.

8 No research relevant to the use of synthetic genomics in plant breeding is under way or is likely to be undertaken in the near future. Therefore, no literature or patent search was carried out, nor was synthetic genomics included in the survey directed at companies applying biotechnology to plant breeding, nor were the changes in the genome or detection issues discussed for synthetic genomics.

3. DEFINITIONS/DESCRIPTIONS OF THE TECHNIQUES

GMOs are defined in Directive 2001/18/EC[9], Article 2 (2)[10]. For the purpose of the Directive a GMO means 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. The Annexes of the Directive include lists of:

1. Techniques which give rise to GMOs such as recombinant nucleic acid techniques, micro- and macro-injection and cell fusion by means of methods that do not occur naturally[11];

2. Techniques which are not considered to result in GMOs such as *in vitro* fertilization, natural processes like conjugation, transduction, transformation and polyploidy induction[12] and

3. Techniques of genetic modification which are excluded from the Directive such as mutagenesis and cell fusion of plant cells which can exchange genetic material through traditional breeding methods[13].

3.1 Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

ZFNs are proteins which have been custom-designed to cut at specific deoxyribonucleic acid (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. The rationale for the development of ZFN technology 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.

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. The possibility of delivering ZFNs directly as proteins into plant cells is currently under investigation.

Three variants of the ZFN technology 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.

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.

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.

9 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 - OJ L 106, 17.4.2001, p. 1–39.

10 For the legal text concerning the GMO definition and relevant annexes of the Directive 2001/18/EC refer to Annex 2 of this report.

11 Annex I A, Part 1 of Directive 2001/18/EC

12 Annex I A, Part 2 of Directive 2001/18/EC

13 Annex I B of Directive 2001/18/EC

3.2 Oligonucleotide directed mutagenesis (ODM)

ODM[14] is another tool for targeted mutagenesis in plant breeding. ODM 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.

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 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.

14 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, oligonucleotide-mediated 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).

3.3 Cisgenesis and intragenesis[15]

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.

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 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.

15 According to the draft report of the NTWG (version 5) it must be demonstrated in the case of transformation through *Agrobacterium* that no T-DNA (transfer DNA) border sequences are inserted along with the gene. Where T-DNA borders or any foreign DNA is inserted, the technique is not considered cisgenesis or intragenesis. However, experts participating in the JRC project usually did not exclude the presence of T-DNA border sequences when using the terms cisgenesis and intragenesis and almost all of the crops derived through cisgenesis/intragenesis reported in literature include T-DNA border sequences. We, therefore, did not exclude these findings from our evaluation. Details are specified in the respective sections.

3.4 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.

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. The epigenetic effect is assumed to decrease through subsequent generations and to eventually fade out, but this point needs further investigation.

3.5 Grafting (on GM rootstock)

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.

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

3.6 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.

The method of reverse breeding includes the following steps:

- Selection of an elite heterozygous line that has to be reproduced;
- Suppression of meiotic recombination in the elite heterozygous line 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 line;
- 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 line.

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[16].

3.7 Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

Plant tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium* sp. containing the desired gene(s) to be expressed in the plant. The genes are locally and transiently expressed at high levels.

The technique is often used in a research context: e.g. to study plant-pathogen interaction in living tissues (leaves) or to test the functionality of regulatory elements in gene constructs. However the technique has also been developed as a production platform for high value recombinant proteins due to the flexibility of the system and the high yields of the recombinant proteins obtained. In all cases, the plant of interest is the agro-infiltrated plant and not the progeny.

Agro-infiltration can be used to screen for plants with valuable phenotypes that can then be used in breeding programmes. For instance, agro-infiltration with specific genes from pathogens can be used to evaluate plant resistance. The resistant plants identified in the agro-infiltration test might then be used directly as parents for breeding. The progenies obtained will not be transgenic as no genes are inserted into the genome of the germline cells of the agro-infiltrated plant. Alternatively, other stored plants which are genetically identical to the identified candidate plant may be used as parents.

Depending on the tissues and the type of gene constructs infiltrated, three types of agro-infiltration can be distinguished:

1. "Agro-infiltration sensu stricto": Non-germline tissue (typically leaf tissue) is infiltrated with non-replicative constructs in order to obtain localised expression in the infiltrated area.

2. "Agro-inoculation" or "agro-infection": Non-germline tissue (typically leaf tissue) is infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant.

3. "Floral dip": Germline tissue (typically flowers) is immersed into a suspension of *Agrobacterium* carrying a DNA-construct in order to obtain transformation of some embryos that can be selected at the germination stage. The aim is to obtain stably transformed plants. Therefore, the resulting plants are GMOs that do not differ from GM plants obtained by other transformation methods.

3.8 Synthetic genomics

Synthetic genomics has been defined as "the engineering of biological components and systems that do not exist in nature and the re-engineering of existing biological elements; it is determined on the intentional design of artificial biological systems, rather than on the understanding of natural biology." (Synbiology, 2006).

Thanks to the technological level reached by genetic engineering and the current knowledge regarding complete genomes' sequences, large functional DNA molecules can now be synthesised efficiently and quickly without using any natural template.

Recently the genome of *Mycoplasma genitalium*, the smallest known bacterial genome, was assembled from commercially synthesised pieces. Synthetic genomics not only provides the possibility to reproduce existing organisms *in vitro*, but the synthesis of building blocks enables the creation of modified natural or even completely artificial organisms.

One of the goals of synthetic genomics is the preparation of viable minimal genomes which will function as platforms for the biochemical production of chemicals with economic relevance.

16 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. For further information see Chapter 5.1.

The production of biofuels, pharmaceuticals and the bioremediation of environmental pollution are expected to constitute the first commercial applications of this new technique.

The NTWG decided to include synthetic genomics in the list of techniques to be evaluated under the current legislation on genetically modified organisms. However, no research relevant to the use of synthetic genomics in plant breeding is under way or is likely to be undertaken in the near future. Therefore, a literature or patent search was not carried out, synthetic genomics was excluded from the survey directed at companies applying biotechnology to plant breeding, and the changes in the genome or detection issues were not discussed for synthetic genomics[17].

17 A comprehensive study on applications of synthetic biology (other than for plant breeding), the Synbiology Project, has been carried out under the Sixth Framework Programme of the EC. We recommend readers who are interested in further information to refer to the report of this project. It comprises an extensive literature and statistical review and an analysis of policies, research and its funding related to synthetic biology in Europe and North America (http://www.synbiosafe.eu/index.php?page=synbiology).

4 RESEARCH & DEVELOPMENT STATE-OF-THE-ART, ADOPTION AND COMMERCIAL PIPELINE

4.1 Research & Development

In order to evaluate the development of research activities and to identify the leading countries and institutions in the field of new plant breeding technologies, we performed a search of a database of scientific publications. 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.

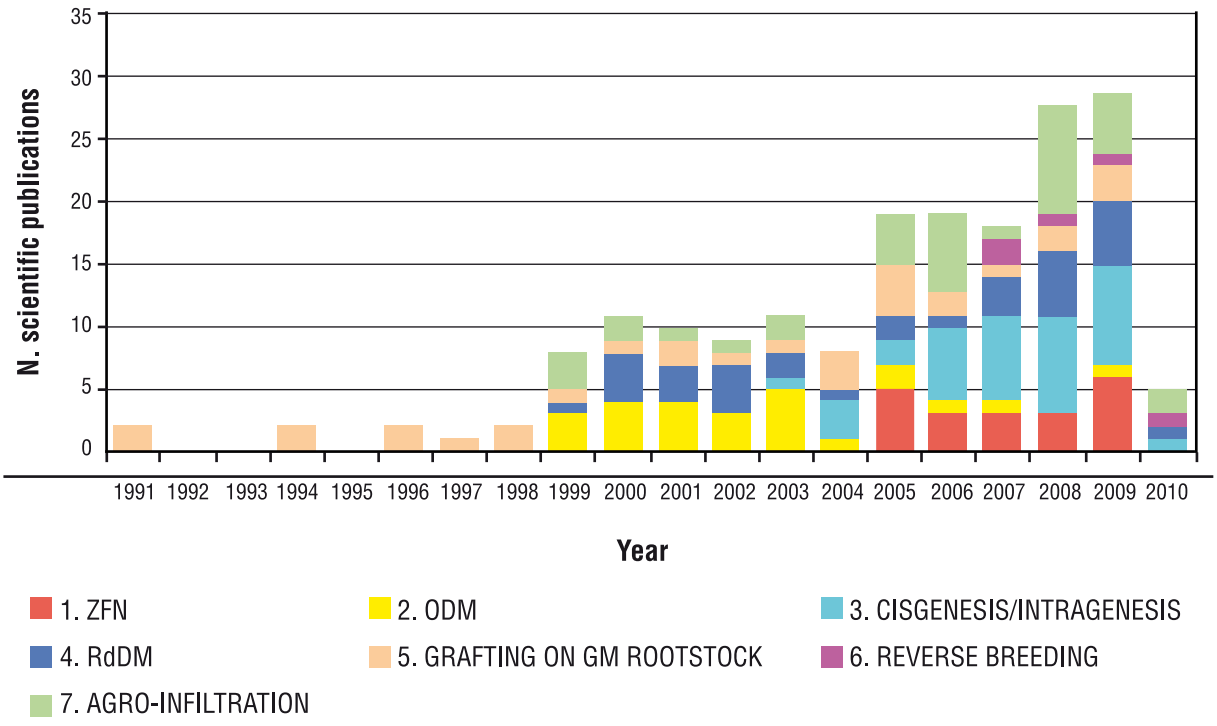
The scientific literature search was performed through a keyword analysis of a database of scientific publications (for information about methodology see Annex 3)[18]. As explained above, synthetic biology was excluded due to the absence of publications related to its application for plant breeding.

A total of 187 scientific publications were identified through the search. Figure 1 shows the distribution over time of the total number of publications identified for each of the seven techniques considered. With the exception of grafting on GM rootstock, all publications on new techniques were produced in the last decade, and the total number of publications is growing, reflecting an increasing level of research activity in the field. The most recent plant breeding technique in terms of publication dates is reverse breeding. The most active technique in terms of growth in number of publications per year is cisgenesis/intragenesis.

Table 1 and figure 2 show the geographical distribution of the publications. According to the results, the EU leads with almost 45% of the publications. Within the EU, the highest number of publications on new plant breeding techniques was produced by the Netherlands (14% of all publications). Detailed, disaggregated data on geographical distribution of publications per technology can be found in Annex 4.

The vast majority of publications (81%) were produced by public institutions, followed by collaborations between public and private institutions (10%) and by private ones (9% of publications).

Figure 1: Development over time of scientific publications on new plant breeding technologies

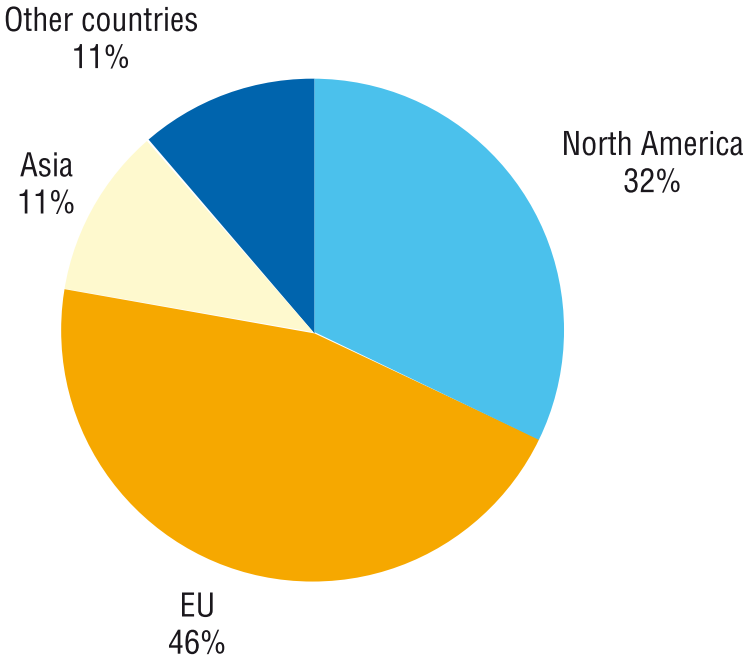


18 The literature search was finalised in April 2010, therefore results include all the scientific publications on new plant breeding techniques until that date.

Table 1: Geographical distribution of scientific publications on new plant breeding technologies

Authors country	ZFN	ODM	CIS/INTRA	RdDM	GRAFT	REV. BREED.	AGRO-INFILTR.	Total	% in total
EU-27	**3**	**10**	**24**	**25**	**20**	**5**	**17**	**104**	**45,6**
Netherlands	1	-	17	4	2	4	3	31	13,6
UK	-	1	3	1	4	-	8	17	7,5
Germany	1	6	1	3	4	-	1	16	7,0
Austria	-	-	-	10	1	1	-	12	5,3
France	1	-	-	3	3	-	4	11	4,8
Italy	-	-	3	1	1	-	1	6	2,6
Belgium	-	3	-	1	-	-	-	4	1,8
Sweden	-	-	-	-	4	-	-	4	1,8
Cz. Republic	-	-	-	2	-	-	-	2	0,9
Finland	-	-	-	-	1	-	-	1	0,4
North America	**17**	**13**	**11**	**3**	**9**	**1**	**19**	**73**	**32,0**
USA	17	12	11	3	8	1	15	67	29,4
Canada	-	1	-	-	1	-	4	6	2,6
Asia	**2**	**2**	**3**	**7**	**7**	**1**	**3**	**25**	**11,0**
Japan	1	2	-	5	1	-	-	9	3,9
Korea	-	-	1	1	5	-	-	7	3,1
China	-	-	-	-	1	1	1	3	1,3
India	1	-	1	-	-	-	1	3	1,3
Bangladesh	-	-	1	-	-	-	-	1	0,4
Thailand	-	-	-	1	-	-	-	1	0,4
Philippines	-	-	-	-	-	-	1	1	0,4
South America	**-**	**-**	**1**	**1**	**1**	**-**	**4**	**7**	**3,1**
Argentina	-	-	-	1	-	-	1	2	0,9
Brazil	-	-	1	-	1	-	-	2	0,9
Peru	-	-	-	-	-	-	2	2	0,9
Cuba	-	-	-	-	-	-	1	1	0,4
Australia	**-**	**1**	**1**	**2**	**-**	**-**	**1**	**5**	**2,2**
Switzerland	**-**	**1**	**3**	**-**	**1**	**-**	**-**	**5**	**2,2**
New Zealand	**-**	**-**	**2**	**-**	**1**	**-**	**-**	**3**	**1,3**
Israel	**-**	**-**	**1**	**-**	**1**	**-**	**-**	**2**	**0,9**
Norway	**-**	**-**	**2**	**-**	**-**	**-**	**-**	**2**	**0,9**
Russia	**-**	**-**	**-**	**1**	**-**	**-**	**-**	**1**	**0,4**
South Africa	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**1**	**0,4**

Figure 2: Geographical distribution of scientific publications on new plant breeding technologies: aggregated results.



The leading institutions on R&D of new plant breeding technologies were identified by analysing authorship of the retrieved publications. Table 2 shows the list of the 10 leading institutions in this field. Considering both the absolute number of publications and the number of techniques investigated, Wageningen University from the Netherlands is in first position. J.R. Simplot Company from the USA is the only private institution appearing in the top 10, and is only involved in R&D of intragenesis.

We then analysed the publications retrieved in order to understand what has been published so far in terms of traits introduced through the new techniques and number and types of plants (model plants or crop plants) on which the new technologies have been employed. This will permit a preliminary comparison of techniques in terms of advanced development and possible applications. Detailed results for each technique are in Annex 4, and a summary is presented below.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

According to the findings of the literature search, the ZFN-1 technique has been used in the model plant tobacco and for mutations in the ALS gene (*acetolactate synthase*) for herbicide tolerance or the reporter genes GUS (beta-glucuronidase gene) and GFP (green fluorescent protein) which are marker genes for selection purposes. For the ZFN-2 technique, publications report its use on the model plant *Arabidopsis* for the mutation of the GUS marker gene. ZFN-3 was used for the integration of the gene PAT (*phosphinothricin phosphotransferase*) that confers herbicide tolerance upon tobacco and maize. The latter represents the only publication of the ZFN technology applied to a crop plant so far.

Oligonucleotide directed mutagenesis (ODM)

More 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 ALS and in maize to mutate the gene AHAS (*acetohydroxyacid synthase*), in both cases to obtain herbicide tolerant plants. Papers also report the use of ODM to mutate the ALS 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 canola) and model plants (*Arabidopsis*).

Cisgenesis and Intragenesis

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 crop plants: potato, apple and melon. Traits introduced into potato include fungal resistance, black spot bruise tolerance and

Table 2: First 10 institutions in the field of new plant breeding technologies ranked according to 2 criteria: absolute number of publications (third column) and number of covered techniques (fourth column: each technique is represented by a letter: Z=ZFN, O=ODM, C=Cisgenesis/Intragenesis, R=RdDM, G=Grafting, B=Reverse Breeding, A=Agro-infiltration). Light blue indicates public institutions and dark blue indicates private institutions.

INSTITUTION	COUNTRY	CITY	N.PUBLIC	TECHNIQUES
WAGENINGEN UNIVERSITY	NL	Wageningen	21	C,R,G,B,A
UNIVERSITY OF CALIFORNIA	USA	Riverside, CA	11	O,R,G,A
JOHN INNES CENTRE	UK	Norwich	9	C,R,G,A
J.R. SIMPLOT COMPANY	USA	Boise, ID	9	C
AUSTRIAN ACADEMY OF SCIENCES	AT	Salzburg	9	R
UNIVERSITY OF AMSTERDAM	NL	Amsterdam	6	Z,O,C,R
IOWA STATE UNIVERSITY	USA	Ames, IA	6	Z
MAX-PLANCK INSTITUTE	DE	Köln	4	O,R,G
UNIVERSITY OF MICHIGAN	USA	Ann Arbor, MI	4	C,Z
INSTITUTE OF PLANT GENETICS AND CROP PLANT RESEARCH (IPK)	DE	Gatersleben	4	O,G

low level of acrylamide production. The technique is used in apple and melon for obtaining fungal resistance.

RNA-dependent DNA methylation (RdDM)

Papers retrieved for induced RdDM report uses in model plants, like tobacco and *Arabidopsis*, and for targeting of model genes (NPTII [neomycin phosphotransferase gene] and GFP). A few publications report the application of RdDM for the modification of the regulation of relevant genes in crop plants such as maize (male sterility), potato (granule-bound starch synthase gene or waxy) or carrots (carrot-leafy cotyledon 1, C-LEC1, an embryo-specific transcription factor) or in ornamentals (flower pigmentation).

Grafting (on GM rootstock)

According to scientific publications, mainly traits for virus resistance have been introduced in GM rootstocks with studies covering potato, grapevine, watermelon, pea and cucumber. Furthermore rootstocks have been genetically modified to achieve improved rooting ability (in apple, rose, walnut and grapevine), tolerance against pests, especially fungi and bacteria (in apple, grapevine, plum and orange), and to improve growth (in watermelon) and osmotic control (in orange).

Reverse breeding

Very few publications have been produced for the technique of reverse breeding to date, only three review papers have been identified and they do not refer to specific crops. Therefore, it is difficult to draw a general conclusion about principally concerned plants by searching scientific literature.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)

More than 300 publications have been identified on two types of agro-infiltration: agro-infiltration "sensu-stricto" and agro-inoculation/agro-infection. Literature results for floral dip have not been analysed further as plants derived from this technique do not differ from GM plants obtained by other transformation methods and therefore the technique is not considered relevant for discussion.

Most publications about agro-infiltration and agro-inoculation report on the use of the techniques for research in model plants, especially tobacco. In particular, agro-infiltration is frequently used to study the interaction of gene products within a living cell, plant pathogen mechanisms or the functionality of regulatory elements. Twenty-six publications have been identified on the use of agro-infiltration for the production of high value recombinant proteins, like vaccines and antibodies. With the exception of tomato, lettuce and white clover that are used in three publications for the production of recombinant proteins, all the other publications describe the use of tobacco plants, especially *Nicotiana benthamiana*. Most recombinant proteins are therapeutic proteins for human disease, i.e. vaccines, antibodies and blood proteins. In a few cases proteins are therapeutic for animals, like bovines, or for plants.

Additionally, 10 publications have been identified on the use of agro-infiltration or agro-inoculation for the screening of pest resistance in plants. Tobacco species are used in three publications, while crop plants like rice, potato, tomato and bean appear in the others. Resistant phenotypes are analysed in potato against the oomycete *P.infestans*, while plant virus resistance is investigated in the other plant species.

Conclusions

In conclusion, what emerged from the literature search is that the field of new plant breeding techniques is very young, as publications started only 10 years ago – with the exception of grafting on GM rootstock (20 years ago) and the number of publications is growing quickly. Public research institutions from European countries have produced the highest number of publications and those from the USA play the second most important role. The proof of concept of the new plant breeding techniques has been achieved by introducing herbicide tolerance and insect resistance traits. While some techniques (like grafting on GM rootstock) have already been tested on many crop plants, others (like ZFN technology) have been tested mainly on model plants.

4.2 Patenting activity in new plant breeding techniques

Intellectual property rights have a fundamental role in the control of exploitation of innovation and in the protection of investments in research. The most important intellectual property rights in plant breeding are plant variety protection rights and patents.

A patent is the sole right for commercial exploitation of an invention. Patentability criteria include novelty, inventiveness and industrial (also agricultural) applicability. They still vary between countries, but harmonisation is increasing due to international agreements. Patenting is a new issue in plant breeding that has been introduced mainly by the application of biotechnology.

Plant variety right is a specific right applicable to new plant varieties which are distinct, uniform and stable. It is the sole right to sell plant varieties for propagation.

A patent search has been performed for the list of new plant breeding technologies established in Chapter 3. The aim of the search was to give an overview of the applications for inventions specifically related to the seven techniques and to identify which companies or institutes have the intellectual property rights on them.

The patent search was performed through a keyword analysis from three public databases: WIPO (World Intellectual Property Organization), EPO (European Patent Office) and USPTO (United States Patent and Trademark Office)[19]. The findings of the patent search were evaluated on the basis of the number of patents per technique. Both patent applications and granted patents were included in the search in order to collect all the information on inventive activity related to the seven new plant breeding technologies. Therefore, we will use the word "patent" to describe granted patents as well as patent applications. Additionally, we did not differentiate between patents with a broad scope and derived patents with a more restricted scope, which would require a more detailed analysis. Each patent listed in the results represents all members of its patent family[20]. Therefore, the number of patents per techniques, as reported in this chapter, corresponds to the number of patent families (for information about methodology, see Annex 5).

As explained above, synthetic genomics was excluded due to the absence of patents related to its application for plant breeding.

A total of 84 patents on the 7 new plant breeding techniques were identified by the search, 70% of them submitted by private organisations, 26% by universities and 4% by a joint collaboration between private and public institutions. The technique for which the highest number of patents have been submitted is ODM (26 patents), followed by cisgenesis/intragenesis and ZFN technology (16 patents each). Grafting on GM rootstock (13 patents) and agro-infiltration (11 patents) follow closely, while for reverse breeding only 2 patents have been identified and for RdDM only 1.

19 The patent search was finalised in November 2010. Patent applications are published 18 months after filing. That means that only patents filed before February 2009 are included in the findings.

20 A patent family is defined as a set of patents - taken in various countries - that protect the same invention (OECD definition).

Figure 3: Development over time of patents on new plant breeding technologies

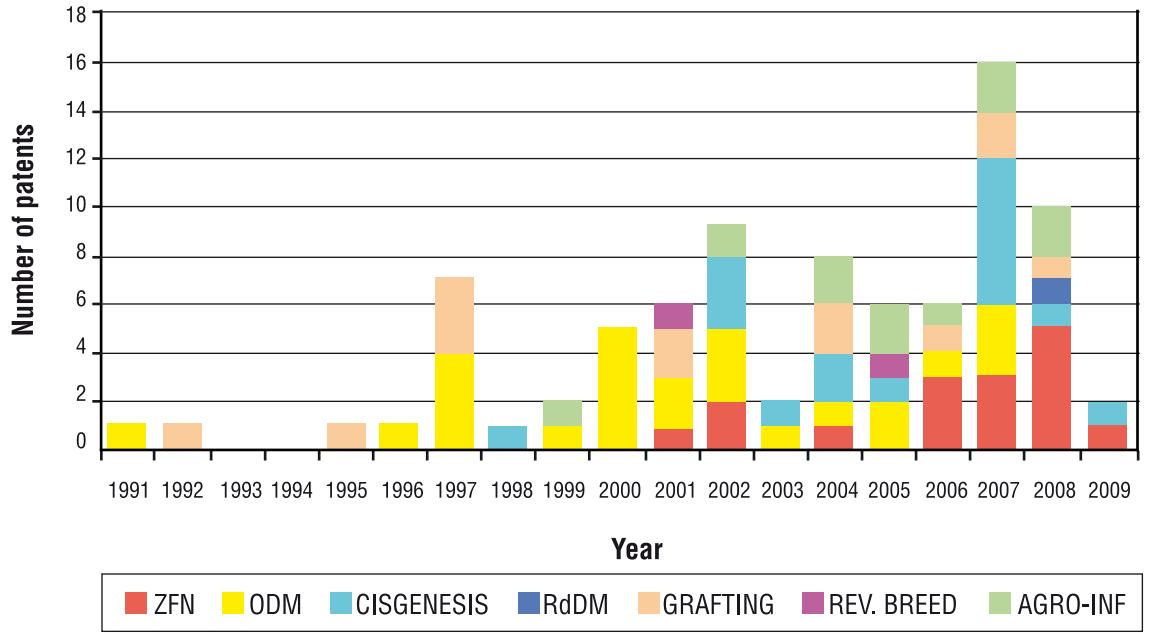


Table 3: Distribution by country of origin of patent assignees on new plant breeding techniques

Assignee country	ZFN	ODM	CIS/ INTRA	RdDM	GRAFT	REV. BREED.	AGRO-INFILTR.	Total	% in total
USA	**18**	**20**	**7**	**-**	**11**	**-**	**6**	**62**	**65**
EU-27	**2**	**6**	**9**	**1**	**-**	**2**	**5**	**25**	**26**
NL	-	4	7	-	-	2	-	13	14
UK	-	1	2	-	-	-	1	4	4
Germany	1	1	-	1	-	-	1	4	4
France	1	-	-	-	-	-	2	3	3
Italy	-	-	-	-	-	-	1	1	1
Israel	**1**	**-**	**-**	**-**	**2**	**-**	**-**	**3**	**3**
Russia	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**2**	**2**
New Zealand	**-**	**-**	**2**	**-**	**-**	**-**	**-**	**2**	**2**
Singapore	**-**	**1**	**-**	**-**	**-**	**-**	**-**	**1**	**1**
South Africa	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**1**	**1**

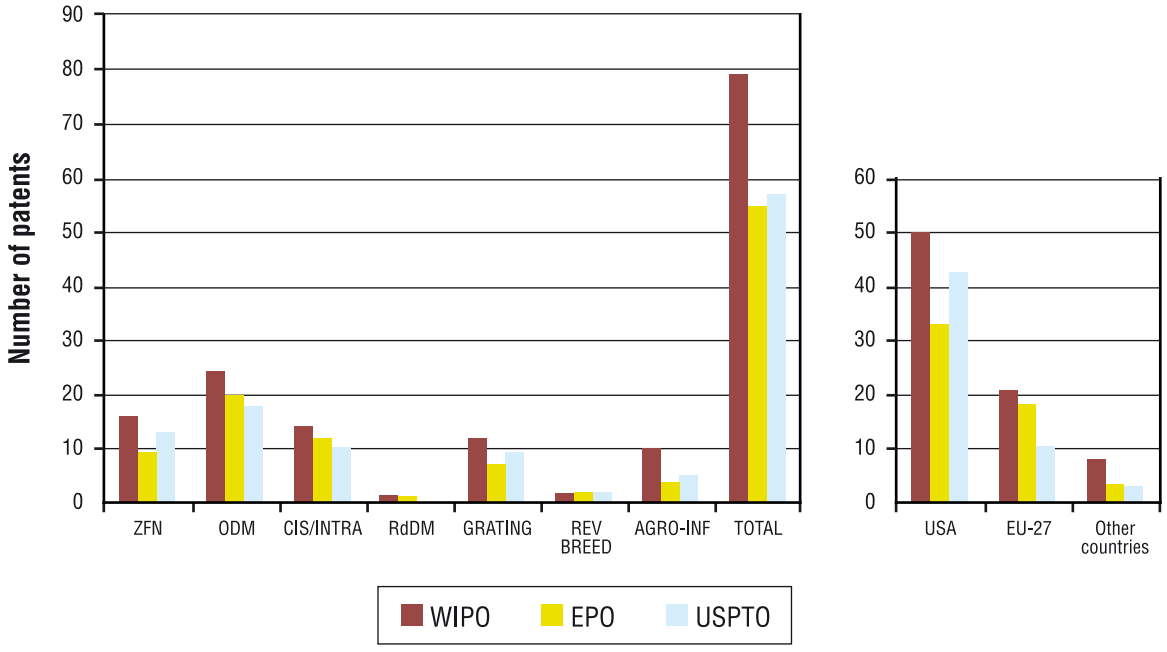
Figure 3 shows the distribution over time of the total number of patents identified for the seven techniques considered. The years reported on the X-axis refer to the priority date (date of first application) of each patent. Like for the literature search results, most of the findings are concentrated in the last decade. According to some studies, patent growth usually follows a trend that is represented by an S-shaped curve, in which the number of patents is low in the initial phase of development of the technology, grows exponentially in the next phase and then, when the technology reaches a maturity phase, reaches a plateau. In the graph of Figure 3 a growing trend is visible, but the number of patents is not high enough to reach a conclusion about the type of curve followed.

The distribution of patent assignees by countries is illustrated in Table 3. According to the results, USA based applicants cover more than half of the total number of patents (65%). The EU is in the second position, contributing to 26% of patents. Within EU countries, the Netherlands is clearly the country that contributes most significantly (14% of the total).

Table 4: Ten leading organisations in patents on new plant breeding techniques ranked according to absolute number of patents (second column on the right) and number of covered techniques (first column: each technique is represented by a letter: Z=ZFN, O=ODM, C=cisgenesis/intragenesis, R=RdDM, G=grafting, B=reverse breeding, A=agro-infiltration). Light blue indicates public institutions and dark blue indicates private institutions.

INSTITUTION	country		TOTAL	TECH
SANGAMO BIOSCIENCES INC	USA	private	11	Z
DOW AGROSCIENCES LLC	USA	private	5	Z
UNIV DELAWARE	USA	public	5	O
SIMPLOT CO J R	USA	private	5	C
CORNELL RES FOUNDATION INC	USA	private	5	G
KEYGENE NV	NL	private	4	O
PIONEER HI BRED INT	USA	private	3	Z, O
CIBUS GENETICS	USA	private	3	O
WAGENINGEN UNIVERSITY	NL	public	3	C
PLANT BIOSCIENCE LTD	GB	private	2	C, A

Figure 4: Patents on new plant breeding technologies at EPO and USPTO, and PCT (Patent Cooperation Treaty) applications (WIPO). (a) Distribution per technique and (b) distribution per geographical origin of the assignee.



An analysis per technology of the USA and EU assigned patents shows the clearly dominant position of the USA in grafting (11 patents versus 0 for the EU), ODM (20 versus 6) and ZFN (18 versus 2). The opposite situation occurs for reverse breeding (2 patents for the EU versus 0 for the USA) and RdDM (1 versus 0), although the number of patents in these techniques is low and they are very recent. A more balanced position is found for cisgenesis/intragenesis and agro-infiltration.

These results are quite different from the findings of the literature search, where the EU has the leading role in terms of number of publications. Despite the strong R&D activities in the EU in the field of new plant breeding techniques, companies and universities in the USA are more active in patenting. This result might be due to the generally stronger tradition of patenting innovation in the USA compared to the EU and differences in the intellectual property systems for plants between the countries. As the plant variety protection right in the USA is weaker, companies and institutes in the USA tend to protect also plant varieties through patents.

From patent search results it emerges that around 50 organisations are active in the field of new plant breeding techniques. Table 4 reports the first 10 organisations in terms of number of patents, 8 of which are private. Most of them are based in the USA, while the others are based in the Netherlands and in the UK. The column on the right shows the techniques covered by the patents of each organisation and we can observe that, with the exception of Pioneer and Plant Bioscience, all of them are specialised in just one technique. J.R. Simplot Company (USA) and Wageningen University (NL) appear in the top 10 in both the patent search and the literature search (see Chapter 4.1, Tab. 1). Although private companies are leading in number of patents, the public sector is also active in patenting related to new plant breeding techniques, particularly in the USA. Indeed, 10 USA public institutions applied for 17 patents out of 53 (32% of USA patents), while in EU only 2 public institutions (Wageningen University and INRA) applied for 4 patents out of 23 (17% of EU patents), 3 of which in collaboration with private companies. This might reflect the stronger habit of patenting inventions by public institutions in the USA than in the EU.

Figure 4 shows the distribution of patent applications at USPTO and EPO and the patent applications that went through the PCT (Patent Cooperation Treaty) route and are administered by WIPO. PCT is a route to obtain protection in any or all contracting states. Within 18 months after the PCT application, the inventor can select the country(ies) in which to protect the invention. As illustrated by Figure 4 (a), the PCT procedure is followed by most applicants in all 7 techniques (94% of total patents). The percentages of patents submitted to USPTO (57% of the total) and EPO (55% of the total) are very similar, even if considering each technique individually. It should be noted that in many cases, the same patent is filed through PCT and after 18 months, both EPO and USPTO are chosen for the protections. The patents following this route appear in all three columns.

Figure 4 (b) illustrates the distribution of patent applications in the patent offices EPO and USPTO for country of origin of the assignee. Additionally, the numbers of patents that followed the PCT route are shown. USA-based assignees applied a higher number of patents in USPTO (43 patents) than in EPO (33 patents), while EU-based assignees applied a higher number of patents (19) in EPO than in USPTO (11). This shows a higher interest of companies and institutes in exploiting the invention in their own country or region and demonstrates that chances for commercialisation of products of new plant breeding techniques are considered similar in both areas.

From the content analysis of each patent, especially focused on the claims, we can distinguish on the one side patents with rather general claims, in which the process of the technique is described without indicating a specific plant species or a specific trait to be obtained, and on the other side patents that claim a specific final product (plant and trait). The following paragraphs give an overview on plants and traits claimed in the patents for each technique. Detailed data on the content analysis of patents can be found in Annex 6.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

According to the patent search, ZFN-3 technology has been patented for its application for the insertion of a sequence of interest in tobacco, *Arabidopsis*, petunia and maize (the only example of a crop plant, similarly to in the literature). Only one patent on ZFN-3 reports a specific trait introduced: male sterility, while the others have more general claims. ZFN-1 and -2 have been patented for their application in tobacco, petunia and maize and mostly for the

attainment of herbicide tolerance. In one patent, the targeted mutagenesis is applied for obtaining plants with reduced levels of phytic acids.

Oligonucleotide directed mutagenesis (ODM)

ODM patents protect its use in tobacco, ornamentals, maize and *Brassicaceae* (such as rapeseed). The main trait for which the technique is patented is herbicide tolerance, but other traits like disease resistance, dehiscence prevention and change in chromatin assembly are also claimed in ODM patents.

Cisgenesis and intragenesis

Patents on cisgenesis and intragenesis cover crop plants and tobacco. Crop plants include wheat and *Solanaceae* like potato and tomato. Traits claimed for cisgenesis and intragenesis are change in composition (e.g. asparagine content in potato in order to reduce acrylamide production in fried potatoes), blackspot bruising tolerance and reduced cold-induced sweetening, and pest resistance in most patents, including fungi and nematodes.

RNA-dependent DNA methylation (RdDM)

One patent concerning RdDM has been identified after a thorough search. It is a very general patent since no specific plant species is claimed. The patent claims that silencing can be directed towards harmful genes for the plant or unwanted traits like over-ripeness.

Grafting (on GM rootstock)

Many different crop plants are covered by patents related to grafting on GM rootstock, like grapevine, apple and citrus or even conifers (i.e. pine trees). The patent search mainly reveals claims regarding rootstocks modified for pest resistance, including resistance to fungi, viruses, bacteria, insects and nematodes. Other applications claimed in patents are the modification of rootstocks' architecture and gene silencing in the scion.

Reverse breeding

Two patents have been identified on reverse breeding. In both cases, 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 seeds, no specific traits are described.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)

Agro-infiltration is often used for research purposes, such as the evaluation of the expression of a transgene in a plant. Therefore, as illustrated in Annex 5, this technique is mentioned in the description of hundreds of patents. In order to restrict the search to specific results, only patents containing agro-infiltration ("sensu stricto" or agro-inoculation) in the claims have been selected. Within them, only patents in which the technique is used for the high level expression of recombinant proteins have been identified as relevant for plant breeding. According to findings, tobacco is the plant claimed in the majority of patents, while other patents claim plants or dicotyledonae in general. Recombinant proteins produced through agro-infiltration include antibodies, vaccines, other pharmaceuticals (e.g. blood proteins) or enzymes (e.g. nucleases and cellulases).

Patents concerning floral dip have not been analysed further as plants derived from this technique do not differ from GM plants obtained by other transformation methods and therefore the technique is not considered relevant for discussion.

Conclusions

In conclusion, patents on the seven new plant breeding techniques have been filed mainly during the last decade and the patenting activity is increasing. Most of the patents can be found in the WIPO database, meaning that applicants have followed the PCT route. A similar number of patents have been submitted to the EPO and the USPTO, suggesting that applicants see commercial interest in the EU and USA markets. However, the large majority of patent applications come from USA-based applicants, mainly USA private companies (65% of all), followed by EU-based applicants (26%). This is in contrast with scientific publications, where the situation is more balanced and in fact the EU leads overall in number of publications. The dominant position for the USA patents is very marked in some of the 7 techniques, such as grafting (11 patents versus 0 for the EU), ODM (20 versus 6) or ZFN (18 versus 2). Another observation is the specialisation of each company in patenting activities in one, or maximum two, of the seven techniques analysed. From our patent search we conclude that the range of crops and agronomic traits protected by the patents is similar to that described in the scientific literature search.

4.3 Current adoption of the techniques by plant breeders and estimated commercial pipeline

The previous chapters have shown that R&D on these plant breeding techniques has been active for 10 years and patenting is also active in all techniques analysed. To ascertain to what extent these technologies have already been adopted by the plant breeding sector and to estimate the status of development of commercial products we carried out a survey of plant breeding companies using biotechnology and of dedicated biotechnology companies. In some cases information on product development was complemented with data obtained during a workshop[21] with participants from the public and private sectors and a search in a database of applications forfield trials in the EU.

Survey description

A survey was carried out in the form of a questionnaire sent to plant breeding companies who use biotechnology and to dedicated biotechnology companies (service providers of the techniques for plant breeders). The questionnaire was sent to 27 companies and 17 completed questionnaires were evaluated. For details on the methodology and the questionnaire see Annexes 12 and 13.

The sample of participating companies covered a wide range from small to big businesses with numbers of employees ranging between 10 and 100 000. Sixty per cent of the participants were individual companies and the others were branches of international groups or part of other complex business structures. Two of the companies were technology service providers and 15 were plant breeders, 5 of which indicated that they were additionally technique providers. In the questionnaire most of the companies mentioned cereals, oilseeds or potatoes as their main crops of interest, and only a few companies focused their business on vegetables.

Companies were asked if they used the new plant breeding techniques studied in this report and listed by the NTWG. (Synthetic genomics was exempted as it is not yet relevant for plant breeding.) Additionally they were asked to specify for which crops and traits the techniques were used and the phase of development of the commercial product. For comparison with the adoption/use of biotechnology in plant breeding in general, companies were also asked about the use of transgenesis and marker assisted breeding. Finally an open question concerning the use of further biotechnological breeding techniques not contemplated in this report was included in the questionnaire.

Adoption by plant breeders and status of commercial development per technology

Each of the seven new plant breeding techniques covered by the survey is being used by two to four of the surveyed plant breeding companies, showing that all of them have been adopted by commercial breeders.

ODM, cisgenesis/intragenesis and agro-infiltration are the most used techniques (by four companies each) and the crops developed with these techniques have reached commercial development phase I-III[22].

From our survey, it appears that the ZFN -1 to -3 techniques, RNA-dependent DNA methylation, grafting and reverse breeding are less used techniques. They are still applied mainly at research level. Detailed information on the situation of the development of commercial products for each technology is given below.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

Plant breeding companies participating in the survey declared applying the ZFN -1 to -3 techniques for breeding maize, oilseed rape and tomato (ranging from research phase to phase III). The traits were not disclosed. ZFN-2 seems to be the least adopted/developed of the three ZFN approaches. During the workshop it was stated that the first crops produced with the help of the ZFN technique could be commercialised within two to three years provided the products are classified as not falling under the GMO legislation.

21 The workshop was organised on 27 and 28 May 2010 in Seville (for the list of participants and the agenda see Annexes 10 and 11).

22 PHASE I: Gene optimisation, crop transformation
PHASE II: Trait development, pre-regulatory data, large-scale transformation
PHASE III: Trait integration, field testing, regulatory data generation (if applicable)
PHASE IV: Regulatory submission (if applicable), seed bulk-up, pre-marketing.

Oligonucleotide directed mutagenesis (ODM)

ODM was declared to have been adopted by four companies participating in the survey with products ranging from phase II to III. Products in phase II to III are mainly oilseed rape and maize varieties with tolerance to herbicides (although general references to other field crops and traits were made).

Cisgenesis and intragenesis

Four companies participating in the survey declared that they were using this technique for breeding crops including maize, oilseed rape (undisclosed traits) and potato (fungal resistance) with products ranging from phase I to III. During the workshop, information on the use of cisgenesis/intragenesis for the breeding (in private and public sectors) of scab resistant apple, potato resistant to late blight (*Phytophtora infestans*) and drought tolerant maize was presented, but the phase of development of products was not specified.

In the case of cisgenesis/intragenesis, information on phase III products could be complemented with an analysis of a database of field trials of GM crops in the EU, maintained by the JRC's Institute for Health and Consumer Protection (referred to as the JRC-IHCP database in this report)[23]. Since cisgenesis/intragenesis involves plant transformation, the hypothesis is that field tests (equivalent to phase III) of these products will be found by searching the GM field trials database. In the database we identified notifications of relevant trials for 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. Additionally, field trials of late blight-resistant potato obtained by the insertion of a gene derived from a wild relative were identified. The marker-free potato only carries the gene from the wild relative together with its own promoter and terminator and the T-DNA borders from Agrobacterium and therefore could be classified as cisgenic.

RNA-dependent DNA methylation (RdDM)

Participants in the survey declared that their companies use RdDM for commercial breeding of maize (at research stage) and oilseed rape (at phase III). Traits were not disclosed.

Grafting (on GM rootstock)

Companies surveyed using grafting on GM rootstocks had products in the research phase or in phase I. Crops and traits were not disclosed. During the workshop it was stated that products are close (five years) to release on the market.

For grafting on GM rootstocks, the JRC-IHCP database of field trials is also of interest since the release of GM rootstocks is covered by the GMO legislation. We identified applications for four different crops concerning grafts onto GM rootstocks: 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. We also identified two notifications for field trials on GM apple trees grafted on non-modified rootstocks.

Reverse breeding

Reverse breeding was declared to have been adopted by companies participating in the survey and/or in the workshop for the breeding of main crops and vegetables, but in all cases at the research stage only.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

Participants in the survey declared that agro-infiltration is used by their companies for research on crops such as potatoes, rape seed and lettuce. For lettuce the aim was to test lines for resistance to downy mildew (*Bremia lactucea*) by inoculation with an *Agrobacterium* strain carrying a *Bremia-effector* gene. In the other cases the traits which the technique was used to select for were not disclosed.

23 For the methodology of the field trial search and the detailed results see Annexes 7 and 8.

Comparative adoption of transgenesis and marker assisted breeding

To compare the adoption of the seven new plant breeding techniques with more established biotechnologies, companies were also asked about their use of transgenesis (classified as giving rise to GMOs) and/or marker assisted breeding (as an example of a breeding technique using biotechnology, but not leading to GMOs). All 15 plant breeding companies participating in the survey indicated the use of marker assisted breeding with crops having already reached the stage of commercialisation. Eighty per cent of the companies also applied transgenesis and crops had mostly reached an advanced phase of development or commercialisation.

Identification of additional new plant breeding techniques not studied in this report

In the questionnaire we included an open question concerning the use of further new breeding techniques not contemplated in this report. Companies mentioned techniques such as dihaploid breeding, double haploid breeding, embryo rescue, genomic assisted breeding, *in vitro fertilization*, polyploidy induction, mutagenesis and cell/protoplast fusion. Many of these techniques have been used for more than 20 years and their classification under the current GMO legislation is clear.

Some companies mentioned in their answers to the questionnaire further new plant breeding techniques. From these techniques, only the adoption of the meganuclease technique is already as similarly advanced as the new plant breeding techniques included in the NTWG list. Two companies declared that they were using the meganuclease technique for the breeding of crops including maize at phase I. Traits were not disclosed.

More information on this topic is available in Chapter 8.2 and Annex 9 which also includes the definitions of these techniques.

Conclusions

Overall, the results of the survey show that that all of the seven new plant breeding techniques have been adopted by commercial breeders. ODM, cisgenesis/intragenesis and agro-infiltration are the most used techniques and the crops developed with these techniques have reached commercial development phase I-III. ZFN technique, RdDM, grafting on GM rootstocks and reverse breeding are less used techniques and are still mainly applied at research level. It is estimated that the most advanced crops are close (2-3 years) to commercialisation (in the event of the techniques being classified as non-GM techniques).

5 DRIVERS & CONSTRAINTS

5.1 Technical/economical advantages and constraints

In principle the commercial development of new plant breeding techniques could be driven by advantages at the technological level (the ability to produce varieties not easily produced with other technologies) or the economic level (lower production costs due to faster breeding process). However, it is also possible to anticipate technical constraints (current efficiency) or economic constraints (costs, including different scenarios for regulatory costs). The section below discusses these possible drivers and constraints based on information obtained in the workshop, the survey of plant breeders, discussions with experts at Wageningen UR, Plant Breeding, NL and from the literature.

Technical advantages

Technical advantages were regarded by most of the companies participating in the survey as a benefit of very high relevance. While the time factor when compared to conventional breeding was rated as of high to very high relevance by the majority of companies, the answers concerning better acceptance by consumers and users compared to transgenesis showed no clear trend. Some companies indicated that consumer acceptance will depend on the classification under the GMO legislation.

Plant breeding is a process lasting up to 15 years (up to 50 years in the case of fruit trees) depending on crop and trait. It starts with the creation of a new genetic variation (if not occurring naturally), followed by selection which involves planting the crops over several years. After the testing and evaluation, the new variety can be multiplied and released. Conventional as well as new breeding techniques are available for the two main steps, creating new variation and selection. New variation can be achieved through crossing, chemical and physical mutagenesis, protoplast fusion and transgenesis, but also by new breeding techniques such as cisgenesis, ZFN technique, ODM or RdDM. Selection can be facilitated by the use of molecular markers, agro-infiltration and cell culture techniques.

Whereas conventional breeding makes use of existing genetic variation within the gene pool of a species or sexually compatible species, the new breeding techniques allow the broadening of the gene pool from which the breeder can select. Like transgenesis, the ZFN-3 technique allows the introduction of long stretches of DNA. Therefore, traits which are not in the gene pool of the species can be introduced.

Some of the new techniques allow site-specific and targeted changes in the genome. Unlike older techniques such as chemical and physical mutagenesis and transgenesis which result in random changes of the genome, the application of ODM or ZFN-1 and -2 leads to site-specific mutations, and ZFN-3 to site-specific insertions.

The use of new plant breeding techniques makes the breeding process faster. Cisgenesis uses the same gene pool as conventional cross breeding, but is much faster by avoiding many steps of back-crossing.

The use of new techniques, especially agro-infiltration provides more accurate selection for genetic traits.

For many of the techniques the genetic information coding for the desired trait is only transiently present in the plants or stably integrated only in intermediate plants. Therefore, the commercialised crop will not contain an inserted transgene[24].

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

The ZFN approach can be used to create site-specific mutations (targeted mutations) which can lead for example to gene inactivation (in the case of the ZFN-1 and ZFN-2 techniques). The ZFN-3 approach can be used for targeted gene addition, gene replacement and trait stacking. Specific gene targeting can prevent so-called “positioning effects” caused by the random insertion of genes in the genome.

The ZFN-1 to -3 techniques are applicable in a wide range of plants including not only main crops but also “smaller” crops such as vegetables provided methods for the delivery of the coding genes into plant cells and regeneration of plants from tissue culture are available. The technique is currently mainly used for the breeding of herbicide resistant crops. A participant in the workshop additionally reported on projects concerning the application of

24 For further information on changes in the genome after application of the new plant breeding techniques refer to Chapter 6.

the ZFN approaches for the removal of antinutrients and allergens through gene knock-out and the removal of antibiotic markers.

Oligonucleotide directed mutagenesis (ODM)

ODM is employed for the targeted, site-specific change of one or a few adjacent nucleotides. Crops with single base changes have already reached development stage, whereas plants with changes of more than one adjoining base pair are still in the research stage.

The technique is regarded as suitable for a broad variety of crops including field crops, such as maize, soy bean and cotton, vegetable crops, asexually propagated crops such as potatoes and bananas, but also for flowers and perennial crops such as fruit trees. Currently ODM is used for obtaining herbicide resistance. These traits offer the advantage of easy selection of plants carrying the mutation. However, ODM can also be used for the introduction of other traits such as prolonged shelf life, pest resistance and for improving quality and health features and yield, and it is expected that crops with these non-selectable traits will reach development stage soon. According to a participant in the workshop, the most advanced applications include starch modification in corn and wheat, benefiting the food processors and consumers, healthier and nutraceutical oils in oilseed crops and industrial oils with new functionalities.

Cisgenesis and intragenesis

Like transgenesis, cisgenesis and intragenesis can be used to insert new genes into plant genomes. However, while transgenesis is used for the transfer of genes from any organism, both eukaryotic and prokaryotic, cisgenesis and intragenesis both deploy DNA fragments from the species itself or from a cross-compatible species. Therefore, the cisgenic and intragenic approach can profit from the same technical advantages as transgenesis. Instead of being technical, the driver for cisgenesis/intragenesis as compared with transgenesis is related to consumer attitudes since the insertion of genes from the species' own gene pool is believed to be more readily accepted by consumers.

A main advantage of cisgenesis/intragenesis compared to conventional breeding is the saving of time necessary for breeding. This is especially important for crops which are vegetatively propagated, such as potato, strawberry or banana, and for crops with long generation times, such as fruit trees. For example, half a century is necessary for breeding of apples with scab resistance. By using cisgenesis or intragenesis, this time can be reduced to five years when isolated resistance genes are available.

Cisgenesis and intragenesis allow the introduction of the gene of interest only, avoiding any linkage drag which is the result of conventional cross breeding. Therefore, a wanted trait can be introduced into high quality cultivars. In conventional breeding many steps of back-crossing are necessary to recover the initial quality of the crop after crossing-in a resistance gene. For crops which are self-incompatible, such as apple, it is not possible to restore the original cultivar by back-crossing.

To achieve durable resistance, several resistance genes need to be introduced into a single crop. Cisgenesis and intragenesis allow inserting stacked genes included in one construct in a single step.

RNA-dependent DNA methylation (RdDM)

RdDM can be used in plant breeding to silence specific genes by the introduction of inverted repeat (IR) sequences and other transgenes that are transcripted into RNAs which are eventually converted into dsRNAs. These dsRNAs lead to methylation of the promoter of the gene(s) to be silenced. In the following plant generation individuals which do not contain the transgene, but which retain the methylated promoter and consequently also the target trait, are selected from the segregants. In this way, modified organisms can be obtained with specific genes silenced but without the transgene in the genome.

RdDM can be used for all crops where a technique to deliver the transgene encoding dsRNA into the cell is available. It can be exploited for modulating endogenous pathways and/or gene activity by modifying the gene expression. RdDM also allows the targeting of multiple genes within a single step which can be used for the creation of dominant traits in polyploid plants.

Grafting (on GM rootstock)

Grafting (of non-GM scions on non-GM rootstocks) is a well established method for many crops. Fruit trees such as apples, but also grape vines, tomato, cucumber and rose plants are usually grafted on

rootstocks. In some cases also interspecific grafts are possible, e.g. eggplant can be grown on tomato rootstocks. The type of rootstock influences the physiology of the scion. For example, dwarf forms of fruit trees can be achieved by grafting on specific rootstocks. However, grafting is not only used for steering the development of the plant but the choice of rootstock also allows the adaptation of the plant to the soil conditions.

The most relevant application in the context of this project is the grafting of non-GM scions on GM rootstocks. Transgenesis can be applied to rootstocks, e.g. to introduce resistance traits against soil-borne diseases or to enhance the rooting ability of reluctant tree species. It is also possible to transform the rootstock with the intention of changing the gene expression in the scion due to the movement of specific proteins and/or RNA from the roots to the scion. In this way a GM rootstock could be used to introduce new traits into a range of genetically distinct scions.

Grafting is also a useful tool for studying the movement of macromolecules in the plant and the silencing and expression of genes.

Reverse breeding

The technique can be used for preserving elite genotypes. Through reverse breeding homozygous parental lines can be produced from a heterozygous plant, which shows the potential of an elite variety. These parental lines can then be crossed to achieve hybrids which reconstruct the heterozygous genotype of the elite plant. With conventional methods it would not be possible to produce a variety which maintains the genotype of such an elite plant.

When applying reverse breeding to a heterozygous diploid, 2^x different DHs can be produced, with x being the basic chromosome number. Consequently, alternative pairs of 'complementary' parental lines can be produced, which when crossed produce the same hybrid variety. Seed production problems in some crops (e.g. cauliflower) can hinder the commercialisation of hybrid varieties. When applying reverse breeding to these heterozygous hybrids it is possible to produce the same variety with two other parental lines, with potentially better reproducibility. This approach is called parental line substitution.

Reverse breeding can also be used to generate so-called chromosome substitution lines. These lines contain one or more chromosomes from one parent in the genetic background of the other parent. This approach can be applied to improve parental lines or for genetic studies for example.

Today homozygous parental lines are usually produced by DH technique within 1-1.5 years. With reverse breeding an additional six months or a year is required for the production of homozygous lines from a heterozygous plant because of the additional transformation step. With conventional breeding, without using the DH technique, 3-10 years would be needed to produce homozygous parental lines.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)

Agro-infiltration is used to transfer a gene construct into cells of plant tissues (mostly leaves) where it is expressed locally and transiently at high levels. In plant breeding, agro-infiltration can be used in the selection step for the optimisation of breeding for disease resistance, e.g. through testing the host reaction to fungal and viral avirulence genes.

Furthermore, agro-infiltration is a useful tool for functional gene analysis, e.g. for studying the functions of genes involved in the biochemical pathways, the interplay of transfer factors or promoters.

Agro-infiltration is a cheap technique, which does not require specific equipment. Results can be obtained within a few days after simply infiltrating plant parts.

Technical barriers

Information on the technical constraints of the new plant breeding techniques comes from the literature search, the survey and the workshop. When asked for the main constraints of the techniques, the companies participating in the survey rated the costs of the technology and the intellectual properties as of high to average and of average relevance respectively.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

Currently ZFNs for approximately half of the 64 triplets coding for amino acids are available. ZFN libraries are being up-dated to improve genome coverage.

The mutation frequency for the ZFN-1 and -2 techniques and the insertion frequency for the ZFN-3 approach reported in different publications vary, but are usually rather low. ZFNs do not always have the desired sequence specificity and affinity because not all of the ZFNs designed and available bind to their cognate DNA triplets in a highly sequence-specific manner. Literature indicates that, given the current state-of-art of the technology, non-specific mutations resulting from non-specific binding of the ZFNs are likely to occur. ZFNs have to undergo a selection and validation process before being commercialised. It is difficult to select plants bearing the expected mutation unless the trait can be used for selection, such as herbicide resistance for example.

The method of delivery into the plant and for the regeneration of plants is crucial for this technique and has to be investigated for each crop case-by-case.

In the cases where ZFN genes are integrated in the plant gene as transgenes, offspring of the transformed plants that still carry the transgenes have to be segregated out. However, also in cases where only transient expression of the gene coding for the ZFN is intended, the possibility of stable insertion cannot be excluded. Therefore, a screening procedure to test for the absence of the ZFN genes is necessary and offspring which still carry the construct coding for ZFNs have to be selected out.

Oligonucleotide directed mutagenesis (ODM)

The mutation rates achieved are usually low and the efficiency of the technique depends on the quality of the synthetic oligonucleotides used. An increase in the length of the oligonucleotides improves the efficiency. Currently oligonucleotides with a length of 20-30 nucleotides are efficiently used, oligonucleotides with a length of 80-100 bp (base pairs) or more are toxic for the cell. Usually a location of the mismatch in the middle of the oligonucleotide results in higher efficiency. Modifications of the oligonucleotides such as the use of locked nucleic acids, methylation or modifications of the ends of the oligonucleotides can be applied to increase the binding capacity and prevent rapid degradation.

The selection of plants bearing the desirable mutation is difficult with the exception of the case of herbicide resistance. However, high throughput screening with sequence based techniques also allows the selection of crops with other traits. The low efficiency of the technique causes logistical problems as a large number of tissue samples have to be handled and consequently the requirement for space in growing chambers is considerable.

ODM has to be applied to protoplasts (unless biolistics are used). The regeneration of the protoplasts requires cell biological expertise and, depending on the type of crop, is regarded as a limiting factor for the application of ODM.

Cisgenesis and intragenesis

Cisgenesis/intragenesis uses the same techniques as transgenesis and consequently has the same limitations. *Agrobacterium tumefaciens* systems which were initially only used for dicotyledonous plants can now also be applied for monocotyledonous crops. The efficiency of the technique ranges from low to high depending on species and cultivar.

With *Agrobacterium*-mediated transformation the vectors used usually contain *Agrobacterium* T-DNA border sequences to facilitate the insertion of the target genes into the plant genome. Therefore, the resulting plants might contain some small, non-coding bacterial border (see also Chapter 6). Direct DNA transfer (particle bombardment or electroporation) can be applied to all crop plants. However, the efficiency is generally low and mostly multiple copies are inserted. Both approaches lead to random insertion in the host genome. The regeneration of plants from tissue cultures or protoplasts causes major challenges for many crops.

The main limitation to the applicability of the technique is the availability of suitable genes from sexually-compatible species that confer useful new properties when inserted in the recipient plants, as the gene pool is more restricted than for transgenesis. However, research in this field is progressing and more genes with interesting properties are being discovered in wild relatives of crop plants.

The concept of cisgenesis allows only the use of the natural regulatory elements of the gene. In the case of intragenesis new combinations of genes and regulatory elements are possible, however all elements have to be derived from the species' own gene pool. Therefore, the use of promoters which are frequently used for transgenesis, and whose function is well understood, is not possible. Plant promoters are composed of several elements (positively or negatively regulating) whose function and interplay is not yet well understood which makes their functioning unpredictable.

The most common approach for selection of transgenic plants is the use of selectable marker genes such as herbicide resistance genes that are introduced into the plant together with the donor gene(s). However, because such selection genes are usually of foreign origin, these selection genes cannot be used for cisgenesis/intragenesis. There are two possibilities to circumvent this problem. Two independent T-DNA vectors can be used: one carrying the gene coding for the wanted trait and the other the gene(s) for the selectable markers. This allows segregating out the marker genes at the end of the breeding procedure. Alternatively, systems are being investigated which use one T-DNA carrying the genes for the trait and the selectable markers, but selectable markers being recombined out in an additional step. This approach leaves behind a recombination site. In the case of gene stacking the presence of multiple recombination sites may cause inter- and intra-chromosomal rearrangements.

RNA-dependent DNA methylation (RdDM)

The biggest hurdle for the commercialisation of crops produced by RdDM is the instability and variability of the gene silencing. The effect is not inherited by 100% of the progeny and is lost after an unknown number of generations. Generally, the degree of silencing is related to the degree of methylation, but this is not always the case. The amount of silencing in the F1 generation can vary by more than a hundredfold and these differences between individuals can become more prominent in progressive generations.

It has been shown that some promoters are more responsive to methylation than others. The knowledge of the functioning of promoters is limited. In particular, it has still to be established which sequences are responsible for up- or down-regulation of gene expression.

Grafting (on GM rootstock)

Grafting on GM rootstock combines two breeding techniques with a long history of use: grafting and genetic transformation. Therefore, the technique is well developed. However, while the influence of different rootstocks on the physical appearance of the scions is known, knowledge of the movement of molecules from the rootstock to the scion and their influence on gene expression in the scion needs to be further investigated.

When grafting non-GM scions on GM rootstocks, 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.

Reverse breeding

Reverse breeding is limited to crops with a haploid chromosome number of approximately 12 or less. With a higher number of chromosomes, the number of non-recombinant double haploids required for finding the complementary pair that reconstructs the original heterozygous plant would be extremely high and not workable.

Reverse breeding is a technically demanding method as both transformation technology and DH technology are employed. Therefore, reverse breeding cannot be used for crops where stable transformation or regeneration of the plant is difficult or impossible to achieve or where the DH technology cannot be applied (e.g. soybean, cotton, lettuce and tomato). Also the efficiency of DH formation of haploids is species-dependent.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)

Applicability of the technique depends on the structure of the leaves. Soft leaves with suitable stomata such as tobacco, tomato or potato can be easily infiltrated, whereas plants with hard leaves are not suitable for the technique.

Although only transient and local gene expression is intended, spreading of *Agrobacterium* and integration of the T-DNA cannot be excluded. Therefore, material from plants which have been infiltrated has to be analysed for the presence of

Agrobacterium and the integration of T-DNA before being used for further breeding.

Barriers related to regulatory uncertainty and costs

When asked for constraints of the techniques, the companies participating in the survey stated that the relevance of the legal situation and the acceptance of consumers and users were unclear and highly dependent on the classification of the techniques under the GMO legislation. The uncertainty of the regulatory status and also the potential level of regulatory requirements and the costs for the approval and registration process, in the event of crops produced using the techniques being classified as GMOs, were additionally mentioned as constraints.

Also, the participants in the workshop raised concern about the regulatory uncertainty of the new plant breeding techniques. These techniques are usually used early in the breeding process which can take up to 15 years. Therefore, due to the unpredictability of the legal situation, it is difficult for a plant-breeder to decide if he should invest in a project using one of these techniques.

Crops obtained by the new plant breeding techniques are not yet commercialised and therefore the economic impact is not known. However, transgenic and conventionally bred crops can be used as a reference. While conventional breeding techniques with low to medium costs for the technique and low registration costs are used extensively in plant breeding, transgenesis, with high costs for the technique, very high registration costs and long delays for approval, is only used for specific projects where breeding has to overcome major challenges. Costs for the new plant breeding techniques range from low (e.g. for agro-infiltration) to high (e.g. for cisgenesis) depending on the technique applied. The registration costs and delays will be low if a technique is classified as non-GMO or very high if classified as GMO. Therefore, the legal status of the new plant breeding techniques will determine if they will be used only in specific projects for the introduction of traits with very high value or extensively for a broad field of applications.

The safety assessment of GMOs is very extensive. It includes the evaluation of substantial differences between GM crops and their non-GM counterparts, molecular characterisation, toxicity and allergenicity studies and the assessment of the environmental impacts and unintended effects. Data requirements are increasing. While data requirements are considerable in other countries such as the USA, Japan and Korea, specific data requirements and especially the long and uncertain timelines cause specific burdens in the EU.

The total costs of bringing a GM plant variety to the market is approximately EUR 70-90 million with costs of EUR 10-15 million for the regulatory package. The time scale for approval is a minimum of 2-3 years worldwide. When the variety is launched in the EU, in addition to the variety approval, a cultivation approval is needed which is expected to take substantially longer.

If, on the other hand, a new plant breeding technique is classified as non-GM, the crop has to only pass variety registration with costs of some EUR 10 000. If launched outside the EU, import registration in the EU is not needed. In the case of a launch in the EU, variety registration will take 2-3 years. Delays in the launch of a new variety due to need for approval under the GMO legislation have major implications for the profit. Launching a variety one year earlier results in an estimated added net present value of EUR 0.7-70 million.

Experience shows that regulatory costs have a strong impact on innovation. An OECD report from 2009 lists the regulatory costs for biotechnology products. Regulatory costs to commercialise GM plant varieties are EUR 0.3-10 million, while those for crops produced using marker assisted selection (MAS) which are classified as non-GMO are estimated at below EUR 7 000. Although MAS is a younger technique than transgenesis, its adoption is already more advanced than the adoption of transgenesis.

Regulation also has a major impact on private research. The percentage of all GM field trials in the OECD carried out by European owned firms decreased in 1999. The same development has been observed in the public sector. The number of field trials carried out by public research is much higher in North America than in the EU.

The high regulatory costs are a burden, especially for small crops, crops with a high number of varieties, special traits, and small and medium companies. The new plant breeding techniques if classified as non-GM techniques could provide an important alternative for sectors where transgenesis cannot be applied because of cost reasons.

Participants in the workshop expressed concern that differences in the regulation of the new plant breeding techniques between the EU and other countries would lead to competitive and technological disadvantages for plant breeders in the EU. This development could cause a brain and technology drain in the sector.

Conclusions

The main driver for the adoption of new plant breeding techniques is the great technical potential of these techniques. Besides the broad applicability in plant breeding, they show specific technical advantages when compared to 'older' techniques. The second main driver for the adoption of new plant breeding techniques is the economic benefit. The use of new plant breeding techniques makes the breeding process faster which lowers production costs.

The main constraints at technical level for the development and adoption of new plant breeding techniques are related to efficiency, which is currently still low for many of the techniques. Therefore, further research and development of the techniques is required. Economic constraints are related to the costs of the technique and costs for the registration, which will be low if a technique is classified as non-GMO or very high if classified as GMO. Therefore, the legal status of the new plant breeding techniques will influence the decision whether to use these techniques only for the introduction of traits in crops with very high value or more extensively for a broad field of applications.

5.2 Background information related to food/feed and environmental safety

Challenges for the commercial development of crops obtained by new plant breeding techniques may stem from safety issues (food, feed or environmental safety). In this section we discuss to what extent safety aspects of the new plant breeding techniques have already been investigated. This chapter is based on the findings of the literature search (described in Chapter 4.1) and additionally on reports on the evaluation of the risks of crops obtained by new plant breeding techniques carried out at national level in EU Member States (MS)[25].

Reports on discussions (at MS level) about food, feed and the environmental safety of the new plant breeding techniques are available from the Netherlands and Belgium. One report (in English) from the Dutch Commission on Genetic Modification (COGEM) from 2006 covers all new plant breeding techniques 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.

One scientific paper from Wageningen University (WUR) in the Netherlands evaluates food, feed and environmental risk of crops derived through all new plant breeding techniques except ZFN technique and RdDM. In addition, we have identified review papers where scientists discuss safety aspects of new plant breeding techniques. Safety aspects are also frequently discussed in the context of research related to technical aspects of the new plant breeding techniques. A small number of reviewed papers relate to research on specific safety aspects of new plant breeding techniques, e.g. the gene flow from GM rootstocks to the soil.

A substantial number of research papers identified in the literature search investigate the efficiency and technical constraints of the techniques as well as intended and unintended changes in the genome of plants obtained by new plant breeding techniques. This information is a prerequisite for carrying out the risk assessment. In the framework of this project three experts evaluated these literature findings. The conclusions of the experts are summarised in Chapter 6 of this report and the full evaluation (which also includes references to the literature) can be found in Annex 15. We have also identified further needs for research into the changes in the genome for these techniques and on their efficiency (see also Chapter 8.2).

25 It is noted that for practical reasons only reports and publications written in English could be taken into account.

Annex 14 provides tables for each specific technique with references to publications and reports identified as relevant for the food, feed and environmental safety of the specific new plant breeding techniques. The tables also include information on the main conclusions or issues discussed for each publication[26]. The reports and publications available for each specific technique are also specified below.

Zinc finger nuclease (ZFN) technology

For the ZFN technique no publications on safety aspects have been identified.

Oligonucleotide directed mutagenesis (ODM)

Discussions of the food, feed and environmental safety of ODM were carried out at national level in the Netherlands (COGEM) and Belgium (BAC) and in a scientific paper from WUR.

Cisgenesis and intragenesis

Food, feed and environmental safety have been evaluated in the Netherlands by WUR and the Institute of Food Safety of Wageningen University (RIKILT). Scientists involved in the research in cisgenesis/intragenesis in the Netherlands, the USA and New Zealand discussed aspects of the risks of the techniques in review papers. Some information can also be found in publications mainly focusing on ethical and societal aspects of cisgenesis.

RNA-dependent DNA methylation (RdDM)

RdDM has so far only been evaluated in the COGEM 2006 report concerning safety aspects.

Grafting (on GM rootstock)

The food, feed and environmental risks of grafting (on GM rootstock) have been evaluated by COGEM and WUR. Three review papers relate to research on gene flow from GM rootstocks to the soil.

Reverse breeding

Safety aspects of reverse breeding were evaluated by COGEM and WUR.

agro-infiltration

The COGEM report and the publication of WUR also discuss safety aspects of agro-infiltration.

5.3 Background information on regulatory issues

As discussed in Chapters 1 and 2 of this report, the classification of the new plant breeding techniques vis-à-vis the current EU GMO legislation is under discussion. Possible constraints due to the high regulatory costs associated with GM varieties for the adoption of the techniques were elaborated in Chapter 5.1. Crops produced using biotechnology are regulated differently in different countries worldwide. Representatives of seed breeding companies participating in the workshop and the survey expressed concern that differences in the regulation of the new plant breeding techniques between the EU and other countries would lead to competitive and technological disadvantages for plant breeders in the EU.

The evaluation of the world-wide regulatory situation for new plant breeding techniques was not an objective of the current JRC project. However, some information on discussions on regulatory issues for specific new plant breeding techniques in the EU or other countries has been identified in the literature search described in Chapter 4.1. Additionally, we took into account reports from discussions on the regulatory status of the new plant breeding techniques in the Netherlands and Belgium (COGEM and BAC).

Annex 14 provides information on publications on regulatory issues[27]. The tables also include information on the main conclusions or issues discussed in each publication. The reports and publications available for each specific technique are also specified below.

26 As food, feed and environmental safety aspects of new plant breeding techniques (see Chapter 5.3) are closely related to the regulatory issues and both topics are frequently discussed in the same publications, we have included all related information in the same table in Annex 14.

27 As food, feed and environmental safety aspects of new plant breeding techniques (see Chapter 5.2) are closely related to the regulatory issues and both topics are frequently discussed in the same publications, we have included all the related information in the same table in Annex 14.

Zinc finger nuclease (ZFN) technology

Discussions on the regulatory issues of ZFN technology, which is one of the youngest techniques covered by this report, have only recently started.

Oligonucleotide directed mutagenesis (ODM)

The classification of crops produced using ODM has been discussed at national level in Belgium and the Netherlands (COGEM and BAC) and in research papers.

Cisgenesis and intragenesis

Compared to the other techniques, the number of publications dealing with regulatory issues of cisgenesis and intragenesis is high.

In the Netherlands, COGEM and RIKILT discussed the regulatory issues together with the environmental and food and feed risks of the technique (see Chapter 5.2). A report compares the regulatory systems in the USA, Canada, Europe, Australia and New Zealand applicable for GM plants and the way they are applied or could be applied to cisgenic/intragenic plants. Additionally regulatory issues of the techniques are discussed by research groups in the Netherlands, the USA and New Zealand in review papers. Further publications deal with the ethical and societal aspects of cisgenesis which are also relevant for regulatory decisions.

RNA-dependent DNA methylation (RdDM)

The very young RdDM technique has only been discussed by COGEM regarding its classification under the GMO legislation so far.

Grafting (on GM rootstock)

The only document explicitly analysing the technique of grafting on a GM rootstock in the framework of the EU GMO legislation is the COGEM 2006 report.

Reverse breeding

As for safety issues, only COGEM has dealt so far with regulatory issues related to reverse breeding.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

To date only COGEM has dealt with regulatory issues related to agro-infiltration (COGEM used the term agro-inoculation for this technique at that time).

6 CHANGES IN THE GENOME OF CROPS CAUSED BY THE APPLICATION OF THE NEW PLANT BREEDING TECHNIQUES

We asked three experts from public administration or public research bodies from different EU Member States to evaluate the changes in the genome of crops caused by the application of the new plant breeding techniques. The experts started their work in March 2010. The new plant breeding techniques, with the exception of synthetic genomics, were distributed between them and the evaluation carried out individually on the basis of papers identified in the literature search. The experts discussed their draft reports in a meeting in July 2010 and several telephone conferences. The evaluation was finalised in September 2010.

The main conclusions of the experts working group concerning intended and unintended changes in the genome for the specific techniques are summarised below. The full text of the report of the experts' working group with references to the literature on which it is based is included in Annex 15.[28]

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

Intended changes/effects

ZFNs are proteins 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.

With the ZFN-1 approach, no repair template is provided to the cells together with the ZFN proteins. The DSB is corrected by NHEJ, which is a natural DNA repair system in the cell. This often results in substitutions to one or only a few bases or in small localised deletions or insertions. The ZFN-1 technique can therefore be used as an efficient mutagenesis method. When these mutations occur in coding regions, they may produce a frame shift, a deletion of one or more amino acids or changes in amino acids, thereby resulting in a high frequency of gene knock-outs.

With the ZFN-2 approach, a continuous stretch of DNA is delivered to the cell simultaneously with the ZFN. This template DNA is homologous to the targeted area, spanning a few kbp, and overlaps the region of the DSB. The template DNA contains the specific base pair alterations to be introduced in the genome by homologous recombination (HR), which occurs at a very low rate in plants compared to NHEJ. The application of the ZFN-2 technique therefore allows the increase of the number of mutations targeted to a certain locus in the gene and the introduction of the base pair(s) of choice compared to random mutations.

With the ZFN-3 approach a recombinant DNA molecule is constructed in which the DNA fragment of the gene cassette of interest is sandwiched between stretches of DNA that are homologous with the DNA sequences flanking the DSB site. This DNA construct, together with the ZFN, is delivered to the cell. Transgene integration targeted to an endogenous genomic locus in the cell can be obtained by HR.

When considering the genomic changes that can be induced for all ZFN approaches, the question is which generation of plants should be considered. If ZFNs are expressed from a vector, the ZFN genes are intended to be transiently present in the cell and are expected to be absent from the final product that will be commercialised. ZFN genes can also be integrated in the plant genome as a transgenic construct. In this case the transgenic ZFN construct would be inherited. Offspring that still carry the ZFN construct would have to be selected out. A screening procedure to test for the absence of the ZFN genes would be a logical part of the selection process.

Unintended changes/effects

The literature indicates that, given the current state-of-art of the technology, non-specific mutations resulting from non-specific binding of the ZFNs are likely to occur. ZFNs do not always have the desired sequence specificity and affinity because not all of the ZFNs designed and available bind to their cognate DNA triplets in a highly sequence-specific manner. They also bind to sites with degenerate sequences leading to non-specific DSBs and consequently to unintended mutations. This can lead to cytotoxicity. Four-finger ZFNs that recognise 24 bp DNA sequences have been shown to promote highly sequence-specific cleavage in human cells. It is therefore hypothesised that four-finger ZFNs would increase specificity compared to three-finger

28 It is noted that the objective of the experts was to evaluate the information on changes in the genome of crops obtained through new plant breeding techniques available in the literature, but not to carry out a risk assessment for these techniques. Therefore, it cannot be excluded that an assessment of the food/feed and environmental safety will identify additional changes or effects as relevant.

ZFNs. Furthermore, sustained expression of ZFNs is likely to contribute to cellular toxicity due to non-specific binding leading to unwanted DSBs in the genome. Inducible promoters could be used to control this problem.

Safety issues

Changes in the genome induced by the ZFN-1 and ZFN-2 techniques can be compared to changes that could occur from natural mechanisms which operate during plant breeding, or from those induced by breeding techniques such as mutagenesis using irradiation or chemical mutagens. The difference is that changes induced by ZFN-1 and ZFN-2 techniques are intended to be site-specific. To date, it is not clear how well this technique works in practice and to what extent off-target effects occur due to non-specific breaks. A point to consider for safety is that with the ZFN technique multiple subsequent site-specific changes may be induced in a single organism, which is not possible by chemical or natural means. Genomic changes produced by the ZFN-3 approach are comparable to those occurring as a consequence of transgenesis. However, since the gene(s) can be targeted to a specific site in the genome, unexpected effects due to so-called 'position effects' are expected to be less in comparison to genetic modification.

Oligonucleotide directed mutagenesis (ODM)

Intended changes/effects

ODM employs oligonucleotides for the induction of targeted mutations in the plant genome. They target homologous DNA and induce site-specific nucleotide substitutions, insertions or deletions through repair mechanisms. If the oligonucleotides and the experimental protocol are adequately designed, the mutation induced by ODM should be highly specific. Organisms developed through ODM cannot be distinguished at the molecular level from organisms bearing the same mutation obtained through mutation techniques such as irradiation or chemical mutagenesis or through selection from natural populations.

Unintended changes/effects

The development of organisms using ODM technology is expected to generate fewer unintentional changes or effects than those generated by breeding techniques based on irradiation or chemical mutagenesis. An advantage of this technology is that it does not use integrative vectors and thus eliminates the risk of any associated insertional mutagenesis. It also acts on specific genes and does not introduce foreign DNA sequences into the target genome. However, the mutation rates achieved are usually low and are comparable to the rate of spontaneous mutations. Therefore spontaneous mutations may obscure the mutations of interest. With the current molecular approaches it is feasible to test for the changes obtained by the mutagenesis in the target locus but it is much more difficult to identify potentially induced mutations at non-specific loci.

Safety issues

ODM does not result in other changes in the genome compared with mutations that occur as a result of natural processes or via irradiation and chemically induced approaches. Potential safety issues (for crops obtained through any of these approaches) may be related to changes in the expression of endogenous genes or to a specific change in the amino acid sequence of an endogenous protein.

Cisgenesis and intragenesis

Intended changes/effects

When applying the cisgenesis/intragenesis technology a DNA fragment from the plant species itself or from a cross-compatible plant species is inserted into the plant genome. In the case of cisgenesis, the inserted gene is unchanged and includes its own introns and regulatory sequences. 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.

Cisgenic and intragenic plants are produced by the same transformation techniques as transgenic plants, e.g. *Agrobacterium*-mediated transformation, following the isolation of genes from the host. Biolistics could also be used. The changes intended when applying this technique relate to modifying the expression of target genes through stable integration in the host genome, as is the case for transgenesis.

With *Agrobacterium*-mediated transformation the vectors used may contain *Agrobacterium* T-DNA border sequences to facilitate the insertion of the target genes into the plant genome. Therefore, the resulting plants might contain some small, non-coding bacterial sequences from the vector such as T-DNA borders. However, specific vectors have been constructed for cisgenic/intragenic approaches which use DNA sequences originating from the same crop species or related species to insert the target genes. These sequences have sufficient homology with *Agrobacterium* T-DNA sequences to allow this function. This approach is termed the P(plant)-DNA approach. Where P-DNA approaches are used, bacterial DNA is absent.

Unintended changes/effects

Irrespective of whether the cisgenic or intragenic approach is used there exists a possibility that the inserts interrupt open reading frames (ORFs) in the host plant or create new ones as a consequence of the insertion process. Deletion of host DNA can also occur following insertion. This could give rise to unintended effects. The same issues are identified as a possible risk for transgenics, for mutation breeding and variation induced by somaclonal variation.

Cisgenic constructs will contain genes and regulatory elements in their "natural" state. Thus similar products could be produced using conventional breeding approaches. However the transfer of such endogenous genes and regulatory elements to another plant could result in modified levels of expression of the target gene(s) and even gene silencing.

As intragenesis uses new combinations of genes and regulatory sequences, gene expression may be changed more extensively (spatially and quantitatively) than with cisgenesis. Furthermore, as intragenic approaches also use RNAi for gene silencing the possibility of effects on other genes and metabolic pathways cannot be excluded. There is therefore the potential for more unintended effects than with cisgenesis.

Safety issues

It has been argued that cisgenesis may be safer than conventional breeding because it prevents the introduction of genes via linkage drag which could lead to unwanted traits (e.g. increase glycoalkaloid content to a higher level than allowed in the regulations for breeder's rights). However, the possibility exists that inserts interrupt known ORFs (which may lead to gene silencing) or create new ones as a consequence of the insertion process (possibly leading to the production of new proteins). Deletion of host DNA can also occur following insertion. Conventional breeding can also result in disruptions to ORFs and other molecular changes including deletions and recombinations. The same can be said for mutation breeding and variation induced by somaclonal variation.

The cisgenic/intragenic approach is based on the assumption of cross-compatibility of the host plant and the plant used to provide the genes. In some cases it could be argued that the germplasm used to source the genes (e.g. a distal wild relative of the recipient plant) may not have a history of safe use in the food chain but this would only be relevant on a case-by-case basis depending on the genes used. The same applies to conventionally bred plants that contain new traits introgressed from wild relatives.

Given that cisgenic/intragenic organisms may contain new proteins, or greatly altered levels of familiar proteins, it has been argued that they generate similar concerns about safety as transgenic organisms.

RNA-dependent DNA methylation (RdDM).

Intended changes/effects

When applying the RdDM technique, genes encoding RNAs which are homologous to plant sequences, like promoter regions, are delivered to the plant cells. These genes, once transcribed, give rise to the formation of small dsRNAs. They induce methylation of the homologous sequences and consequently inhibit their transcription.

The efficiency of silencing can be up to 90% and is dependent on the active transcription of the promoter. Generally, the degree of silencing is related to the degree of methylation, but this is not always the case. The amount of silencing in the F1 generation can vary by more than a hundredfold and these differences between individuals can become more prominent in progressive generations. Silencing and differences in silencing have been observed to be transmitted to at least the F3 generation.

Promoters of endogenous genes appear to be less amenable to silencing than transgene promoters. Cytosine content and local DNA features have been proposed as factors affecting RdDM in plants. Methylation is restricted to the region of sequence homology with the dsRNA. No spreading of methylation into sequences flanking the region of homology between the IR RNA (also known as hairpin RNA (hpRNA)) and the target DNA has been observed.

When the template RNA for dsRNA is introduced by transfection or by a vector system, the templates are intended to be present only transiently in the cell and are expected to be absent from the final commercialised product. When an RNAi construct is used, commercial products lacking the construct can be obtained by segregation. In all cases a screening procedure to test for the absence of this construct would be a logical part of the selection process.

Unintended changes/effects

It is not clear for how many generations the effect of gene silencing by RdDM remains in the absence of the inducing construct. An unintended effect could therefore be the loss of silencing of the specific gene in the commercial product. Another potential unintended effect could be the silencing of genes with homologous promoter sequences. Alternatively, the production of other small RNAs from an hpRNA can occur that may regulate the expression of other genes not intended to be manipulated.

Safety issues

RdDM is not expected to cause changes in the genome other than DNA methylation. Methylation of DNA is a natural phenomenon and can be induced by environmental conditions and by traditional breeding. This is illustrated by the fact that methylation is widespread in plant chromosomes. Indeed, approximately 20% of the *Arabidopsis* genome is methylated. Potential safety issues may therefore only be related to changes in the expression levels of targeted endogenous genes.

Grafting (on GM rootstock)

Intended changes/effects

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 regard to plant breeding, the grafting of a non-GM scion onto a GM rootstock is considered to be the main approach. However, it is also possible to graft a GM scion onto a non-GM root stock and indeed a GM scion onto a GM rootstock.

Should both the rootstock and scion be transformed using methods known to modify the genome then the entire plant is considered to be GM. Should a GM scion be grafted onto a non-GM rootstock then clearly above ground parts such as seeds, edible components etc. will be transgenic. If only the rootstock is transformed then intended changes to the genome are targeted at root tissues.

Intended changes will be dictated by the selection of promoters and gene sequences which are targeted for modified expression, as would be the case for a "standard" transgenic plant. However, it is conceivable that there might be an intention to transform only the rootstock with a view to changing protein or gene expression in the scion due to the movement of specific proteins and/or RNA from the roots to the scion. In this way a GM rootstock could be used to introduce new traits into a range of genetically distinct scions.

Unintended changes/effects

One consideration is whether or not mechanisms exist for the transmission of nucleic acids, proteins or other metabolites which could induce changes to the genome in the non-transformed tissues following grafting. With respect to the possible movement of DNA between rootstock and scion which could result in genome changes in the scion there is little evidence that this is an issue. Also the transfer of plastid genetic information in a graft from rootstock cells to the cells of the scion and vice versa has been reported. Chimeric cells were recovered from the graft site but it was not clear if the genetic information was transferred as DNA fragments, as an entire plastid genome or as plastid. Genetic exchange appeared to be restricted to graft sites only (flowers and fruits from a non-GM scion did not contain GM DNA

sequences from the GM rootstock). Therefore, one could conclude that unintended changes to the coding sequence of a non-GM scion grafted onto a GM rootstock do not occur.

With regard to unintended effects resulting from the transmission of other macromolecules from root to scion, it is known that recombinant proteins, hormones and non-coding RNA (e.g. siRNAs (small interfering RNA)) can be transported from the GM rootstock of a graft to the scion where they can induce an effect. It is known that RNAi can lead to RNA-directed DNA methylation of promoter regions, resulting in modified expression of the target genes (see section on RdDM above). So, although the resulting offspring from a graft can be regarded as non-GM, mitotically and meiotically heritable (epigenetic) changes in gene expression that do not involve a change in the DNA sequence can still occur. Also the finding that non-transgenic *Nicotiana benthamiana* grafted on a rootstock expressing a *Grapevine virus A* (GVA) minireplicon became resistant to GVA infection with 70-90% efficiency has been reported.

Safety issues

The major issue relates to any unintended changes in gene, protein and trait expression in the scion resulting from unwanted movement of proteins and RNA from GM roots to non-GM scions.

Reverse breeding

Intended changes/effects

The intended goal of the technique is to generate perfectly complementing homozygous parental lines through a suppression of meiotic crossovers and the subsequent fixation of non-recombinant chromosomes in homozygous DH lines. In this respect, there are no changes foreseen in the genome of the selected non-GM offspring.

Unintended changes/effects

To date there are very few publications on reverse breeding. Unintended effects could include the silencing of other homologous sequences in the genome as a result of the presence of the RNAi construct. This would not induce genomic changes, but could affect expression levels. Another unintended effect of the technique could be an incomplete suppression of meiosis. This would lead to some degree of meiosis and recombination, which are natural processes in plants.

Safety issues

Silencing of other homologous sequences in the genome by the RNAi construct could affect expression levels, which can also occur under natural conditions. Suppression of meiosis, incomplete or not, can also be obtained by chemical and physical means or by environmental factors.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

Intended changes/effects

Depending on the tissues and the type of constructs infiltrated, three types of agro-infiltration can be distinguished:

1. "Agro-infiltration sensu stricto": Non-germline tissues are infiltrated with a liquid suspension of *Agrobacterium sp.* containing a genetic construct in order to obtain localised expression in the infiltrated area.

2. "Agro-inoculation" or "agro-infection": Non-germline tissues (typically leaf tissues) are infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant.

3. "Floral dip": Germline tissues (typically flowers) are infiltrated with a DNA-construct in order to obtain transformation of some embryos that can be selected at the germination stage.

The intended goal of the technique is the transient and temporary expression of specific coding sequences without integration of the introduced DNA in the plant genome. However, in the case of the floral dip it the aim is to obtain stably transformed seedlings without the need for a plant cell regeneration phase. The resulting plant has the same properties as a transgenic plant.

Unintended changes/effects

While the aim is the transient and temporary expression of a coding sequence, the integration of T-DNA fragments into the genome of cells in the infiltrated area cannot be ruled out. This is true for agro-infiltration and for agro-inoculation/ agro-infection. In the case of agro-inoculation/ agro-infection, the spreading of the gene construct introduced into the viral genome is caused by systemic spreading of RNA viruses throughout the plant via plasmodesmata. Since the gene construct is spread via RNA molecules, they do not integrate into the plant genome.

Safety issues

Agro-infiltration is used to screen for genotypes with valuable phenotypes that can then be used in breeding programmes. For instance, agro-infiltration with specific genes from pathogens can be used to evaluate plant resistance and the mechanisms underpinning the resistance. The most resistant plant identified from the actual agro-infiltration study might then be used directly as a parent for breeding but the progenies obtained will not be transgenic as no genes are inserted into the genome. Alternatively, if possible, other plants which are genetically identical may be used as parents.

Progeny plants obtained after a floral dip treatment that has inserted the DNA fragment in the genome do not differ from GM plants obtained by other transformation methods.

7 POSSIBILITIES FOR DETECTING AND IDENTIFYING CROPS PRODUCED WITH NEW PLANT BREEDING TECHNIQUES

Availability of detection methods is a regulatory requirement for the approval of GMOs under EU legislation. It was therefore decided that the possibilities for detecting crops produced with new plant breeding techniques should be investigated. The findings are described as part of this report.

For this investigation we established a "New Techniques Task Force" (NTTF). In order to benefit from the expertise already existing on GMO detection and analysis within the European Network of GMO Laboratories (ENGL[29]), eight technical experts were selected from amongst the ENGL members to join the NTTF.

Between April and November 2010, the NTTF held 11 conference calls and 3 meetings (including a meeting with industry representatives in November 2010). In December 2010, a NTTF report on "New Plant Breeding Techniques and Challenges for Detection and Identification" was produced. This technical report is summarised below and a full version of the report is included in Annex 16.

For this evaluation the NTTF agreed in particular to:

- focus on technical issues related to detection and identification of genetic modifications resulting from new plant breeding techniques (i.e. not to include discussions on future regulatory decisions on new plant breeding techniques).
- focus on the list of new plant breeding techniques addressed in the NTWG, with the exception of synthetic genomics which is not yet relevant for plant breeding, and therefore to focus on the following seven techniques:
  1. Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
  2. Oligonucleotide directed mutagenesis (ODM)
  3. Cisgenesis and intragenesis
  4. RNA-dependent DNA methylation (RdDM)
  5. Grafting (on GM rootstock)
  6. Reverse breeding
  7. Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)
- focus on the analysis of crops developed (i.e. not taking into account processed products and mixtures thereof).
- focus not only on the detection of a genetic modification but more importantly on the identification of the genetic modification as intentionally introduced by a new technique.

Enforcement becomes more difficult if the resulting organisms are indistinguishable from their conventional counterparts or natural variants and cannot be detected as being the result of a genetic modification technique. Therefore, the NTTF decided to make an important distinction between the concepts of "detection" and "identification" which should be understood, for the purposes of this NTTF report, as follows:

DETECTION: detection of a genetic modification means that it is possible to determine the existence of a change in the genetic material of an organism (for instance at the level of DNA through the presence of a novel DNA sequence) by reference to an appropriate comparator.

IDENTIFICATION: identification of a genetic modification means that it is possible not only to detect the existence of a change in the genetic material of an organism (see detection text before) but it is also possible to identify the genetic modification as one that has been intentionally introduced by a new technique.

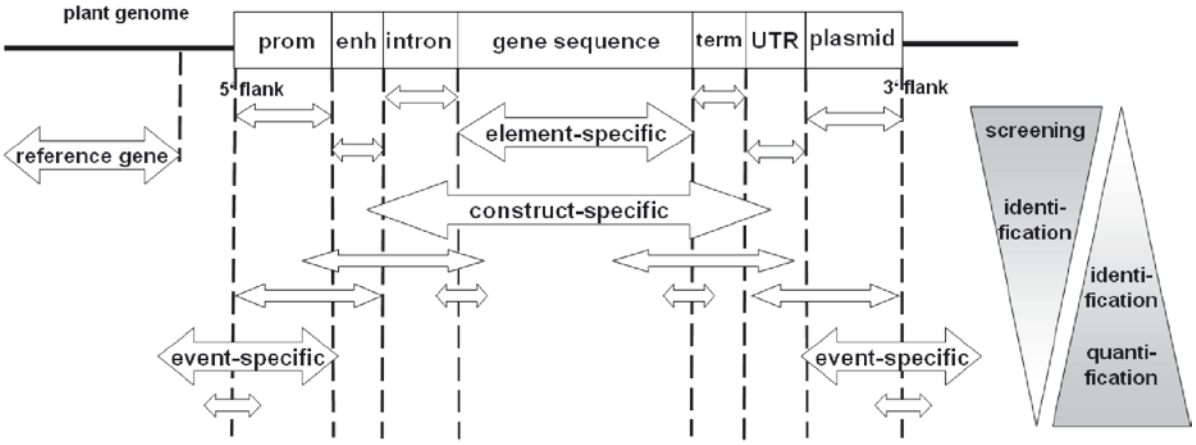
For each individual new technique, the NTTF also agreed to consider the following two scenarios:

WITH PRIOR KNOWLEDGE: refers to cases where information is available (for instance at the level of DNA sequence) on the product resulting from the use of a new plant breeding technique. This information may be made available for instance by the company having developed the product.

WITHOUT PRIOR KNOWLEDGE: refers to cases where no information at all is available on the product resulting from the use of a new plant breeding technique. This situation may be compared with the challenges already raised today regarding the detection of "unknown" GMOs.

29 The ENGL is a consortium of national reference laboratories (including around 100 members) which was established by Regulation (EC) No 1829/2003 on GM food and feed and which is assisting the European Union Reference Laboratory for GM food and feed (EU-RL GM FF) in its duties, in particular with the validation of GMO detection methods.

Figure 5: Schema of a transformation construct comprising seven elements inserted into a plant genome through a certain transformation event and, therefore, flanked by specific DNA sequences of the plant genome.



Arrows of the upper four rows indicate regions suitable for element-specific detection. Such screening assays target widely used genetic elements like promoters.
Arrows in the following three rows in the middle indicate regions suitable for construct-specific detection. Construct-specific assays are designed to comprise a junction between different elements of the inserted sequence.
Arrows in the two rows at the bottom indicate regions suitable for event-specific detection. Event-specific assays are the most specific ones and are constructed over a junction between the host and the inserted sequences, with specific primers for the inserted gene and the flanking genomic sequence.
An example for a reference gene is indicated. The two triangles at the right hand side indicate a gradient of suitability for screening, identification, and quantification.

7.1 State-of-the-art for detection and identification of genetic modifications in plants

Information concerning the genotype of plants can be obtained at different levels, e.g. at the level of DNA, proteins and metabolites. Modern analytical methods exist on all of these levels and the NTTF discussed their applicability for the detection and identification of crops developed through new plant breeding techniques.

This section was developed using existing knowledge and information on the techniques available for GMO detection. In particular it is based on the activities of the EU-RL GMFF and of the ENGL, as well as on the activities of standardisation bodies like ISO and CEN.

The conclusions regarding the state-of-the-art for detection and identification of genetic modifications can be summarised as follows:

DNA-based analysis

DNA amplification-based methods (PCR)

Amplification techniques involve denaturation of the double-stranded nucleic acid followed by the annealing of a short oligonucleotide (primer) and primer extension by a DNA polymerase. The most common technique is the polymerase chain reaction (PCR) technique, employing a thermo-stable DNA polymerase. PCR is the most commonly used technique for GMO detection. Figure 5 details the different levels of specificity of GMO detection possible with PCR technology (from screening to construct-specific and event-specific) depending on the type of DNA sequence information available.

Any PCR-based method relies on the availability of a certain minimum of information about the target DNA sequence. Some information needs to be known about the inserted DNA sequence and about the 5' and/or 3' neighbouring genomic DNA sequence in order to allow the identification of an intentional genetic modification (see further details below).

Without prior knowledge, reliable identification of a genetic modification is not possible even with the most sophisticated available methods for DNA analysis.

PCR-based analytical methods for the detection of intentionally modified DNA sequences provide high sensitivity and specificity. PCR supports the development of specific methods that allow the detection as well as the identification of intentionally modified DNA, i.e. plants with known intentional modifications can be differentiated for instance from plants presenting similar phenotypes and from plants possibly presenting a similar DNA modification through natural mutation.

Insertions larger than 80 bp

For the detection and the identification of an insert, the primers and probe need to be designed within the insert. Large inserts can be detected and identified when at least 80 bp of the inserted sequence is known.

For event-specific identification, a sufficient part of the sequence of the insert as well as a part of the adjacent sequence must also be known, in order to be able to design an event-specific primer pair and a probe. This information is a prerequisite for the unambiguous identification of an intentional genetic modification.

Short insertions

PCR-based methods are also capable of detecting and identifying short insertions of less than 80 bp. In this case specific primers are designed in order to bind to sequences including the insert and its flanking regions sites or to bind only to sequences directly flanking the insert. Irrespective of the number of modified base pairs, the specific primers should be at least approximately 20 nucleotides long and specific in sequence for the modification and its direct vicinity. In order to identify a short intentional modification and to differentiate it from a possible natural mutation, information on the modified sequence and the nucleotide sequence in its direct vicinity is required for the design of the specific primers.

Modification of one or a few nucleotides

Intentional modifications of a single or a few nucleotides can in principle be detected. Information on the site of the modification and the nucleotide sequence in its direct vicinity of approximately 20 bp (including the site of modification) is necessary to in theory ensure the uniqueness of the sequence forming the newly created junction in the genome. For the amplification of this unique sequence by PCR further information upstream and downstream is required for the design of primers. If this 20 bp string matches a repetitive sequence in the genome however it cannot unambiguously characterise the location of the modification.

Deletions

Deliberate modifications by deletions can also be detected in a similar way to that described for modifications by short insertions. Information on the site of the deletion and the nucleotide sequence in its direct vicinity of approximately 20 bp including the site of deletion is necessary to in principle ensure the uniqueness of the sequence forming the newly created junction in the genome. For the amplification of this unique sequence the same requirement applies as for the modification of a single or a few nucleotides. If this 20 bp string matches a repetitive sequence in the genome however it cannot unambiguously characterise the location of the modification.

DNA Sequencing

DNA sequencing allows the order of the nucleotide bases adenine, guanine, cytosine and thymine in a DNA strand to be determined.

The detection of intentional modifications by DNA sequencing also requires prior knowledge of the nucleotide sequence of the introduced modification and its vicinity, as described for DNA amplification-based methods (most of the DNA sequencing techniques also include a PCR DNA-amplification step).

Developments in the field of DNA sequencing are rapidly expanding. However it can be concluded that to date whole genome sequencing is not applicable for routine analyses of genetic modifications (in particular, analysis of the huge amount of data generated is still challenging and costs are also still relatively high).

DNA hybridisation-based methods

Hybridisation-based methods rely on the fact that a DNA double helix molecule will become single-stranded at an elevated temperature. At a temperature below its “melting point” the two complementary nucleotide sequence strands will fuse (hybridise) to each other as soon as they meet at complementary stretches of sequence.

The detection of intentional modifications by hybridisation-based methods also requires prior knowledge of the nucleotide sequence of the introduced modification and its vicinity, as described for DNA amplification-based methods.

All in all, it can be concluded that DNA hybridisation methods are not practical for routine analyses of genetic modifications (in particular, DNA hybridisation techniques offer low sensitivity compared to amplification-based methods).

Protein-based analysis

The genetic information in a plant (DNA) is translated into proteins via an intermediate (RNA). Proteins are made up of amino acids. Each amino acid is specified by a triplet code of the DNA and transcribed RNA. The sequence of amino acids specify the three dimensional structure of the protein and also its functionality, although some changes can occur after the production of the protein and are referred to as post-translational modification.

Proteins in plants can, for example, act as enzymes driving the metabolism of the cell: respiration, photosynthesis, gene replication, etc., or act as structural proteins.

Application of protein-based methods will only be possible when the following prerequisites are fulfilled:

- Prior information on the new protein or on the protein modification/amino acid change is required to be able to apply protein-based methods.

- Protein-based methods require intact proteins in sufficient quantity, so processing of the material reduces or completely excludes their applicability.

- The detection of a change in the protein would not always enable identification of a specific genetic modification. In general, a protein-based detection method will only be useful where the genetic modification creates a novel or changed protein (e.g. post-translational modification) or removes a protein product. It is anticipated that in most modifications this will be the case as the aim of the modification will be to change some function in the plant.

Immuno-based methods, like Lateral Flow Devices (LFD) and Enzyme Linked Immuno Sorbent Assays (ELISA), are particularly useful for routine use in detection (and possibly identification) of genetic modifications but the development of the required antibodies involve some investment in research and development. Protein sequencing, electrophoresis and western blots are less useful for the analysis of many samples on a routine basis.

Metabolite-based analysis

Metabolites are substances produced by the metabolism of the plants. Metabolites encompass a wide range of chemical compounds. Primary metabolites are required to maintain the functioning of the cell for processes such as photosynthesis or respiration. Secondary metabolites have a function in the plant.

A process of genetic modification is expected to change the metabolite profile of an organism when compared to the wild type. The metabolite pool from an organism is called the metabolome and its study is called metabolomics.

The most powerful of the metabolite-based techniques are Nuclear Magnetic Resonance (NMR), Gas Chromatography – Mass Spectrometry (GC-MS) and Liquid Chromatography – Mass Spectrometry (LC-MS). Each technique has its own merits. To ensure maximum coverage of metabolites, parallel studies implementing all techniques are advised. The strength of the techniques is in screening for unexpected effects.

Where significant differences are determined (either differences in concentrations of metabolites, or presence of novel metabolites) they form the basis of metabolite-based detection strategies. Once known, these differences can be determined using simpler analytical techniques so that more cost effective routine screening can be performed.

To use any of these techniques there would be a significant need for methodological development to make the techniques reproducible and non-selective. The techniques need to be: sensitive (MS is better than NMR), reproducible (NMR is better than MS), and have the ability to elucidate structure (NMR and MS can both do this). Also it is necessary to improve statistical analysis to find out which analytes are significant and robust biomarkers of differences.

However, metabolite-based methods alone would not be able to detect, identify or differentiate plants modified with a specific genetic modification technique from similar plants produced using a different technology. They may be used in combination with other techniques to detect or identify plants modified with a specific genetic modification technique.

General conclusions on detection and identification of genetic modifications

To date, a broad range of methods can be applied to detect genetic modifications, including DNA-based methods, protein-based methods and metabolite analysis.

Based on the review of this large diversity of methodologies, the NTTF considers that:

- DNA is the ideal target molecule for unambiguously detecting and identifying a change in the genetic material of an organism as the intended result of a genetic modification technique.

- DNA-based methods are the most appropriate for detection and identification of genetic modifications and potentially offer all required levels of specificity and ability to quantify the target i.e. a specific DNA sequence (protein-based methods or metabolite analysis methods in particular have some limitations in terms of identification of a change as the intended result of a genetic modification technique and of differentiation from natural mutation).

- Within DNA-based methods, DNA amplification-based methods (PCR) are the most appropriate for detection and identification of genetic modifications (DNA-sequencing methods in particular have some limitations in terms of practical application for routine analysis while DNA-hybridisation methods have some limitations in terms of sensitivity).

However, any PCR-based method relies on the availability of a certain minimum of information about the target DNA sequence. Some prior information about the inserted DNA sequence is necessary and about the 5' and/or 3' neighbouring genomic DNA sequence in order to allow the identification of an intentional genetic modification.

Without prior knowledge, reliable identification of a genetic modification is not possible even with the most sophisticated methods available for DNA analysis.

7.2 Specific considerations for detection and identification of intentional genetic modifications by new plant breeding techniques

Based on the previous section, the NTTF comes to the general conclusion that DNA amplification-based methods (PCR) are the most appropriate for detection and identification of genetic modifications.

The EU regulatory approach based on validation of GMO event-specific PCR methods can be considered as the "reference" or "baseline" for detection and identification of products obtained through a deliberate genetic modification technique, be it through genetic engineering (like GMOs defined under Article 2 (2) in conjunction with Annex IA Part 1 of Directive 2001/18/EC) or through a new technique.

In this section we report the possibilities of detection and identification for each of the seven individual new plant breeding techniques. Based on the current available detection methods summarised before, the "reference" or "baseline" for this analysis is therefore the PCR-based approach for detection of GMOs (known or unknown).

For each specific new plant breeding technique the following information is given:

1. **Definition of the individual new technique (including, if necessary some general considerations)**

2. **Detection and identification with prior knowledge**

This scenario refers to cases where information is available (in particular at the level of DNA sequence) on the product resulting by the use of a new plant breeding technique. This information may be made available for instance from the company having developed the new product (plant). Cross-reference is made to Chapter 7.1 which includes details on the type of information required to permit the detection and identification of genetic modification.

3. Detection and identification without prior knowledge

This scenario refers to cases where no information at all is available on the product resulting from the use of a new technique. It is to be noted that in the case of "unknown" GMOs (i.e. GMOs for which no information is available, for instance because no regulatory application has been filed,) detection and identification are challenging[30].

4. Conclusions

The conclusions summarise the opinion of the NTTF regarding the possibility to detect and more importantly to identify products from the various individual new plant breeding techniques i.e. the possibility to differentiate them from products resulting from natural mutations or obtained from other breeding techniques, such as mutagenesis.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

ZFN-1 and ZFN-2

For organisms modified by the ZFN-1 and ZFN-2 techniques (leading to small modifications) detection with DNA-based methods would be possible provided some prior information on the introduced modification is available. But identification will not be possible because ZFN-1 and ZFN-2 products cannot be distinguished at molecular level from products developed through other mutation techniques or occurring through natural mutations (see Chapter 7.1 Modification of one or a few nucleotides).

Without prior knowledge, detection of small modifications introduced by ZFN-1 and ZFN-2 would be demanding and unlikely to be used in routine laboratories. Identification will not be possible.

ZFN-3

Detection and identification of organisms modified by ZFN-3 technology (leading to large modifications) are possible through the amplification-based methods (PCR) currently used for GMO detection, with the prerequisite that prior adequate DNA sequence information on the introduced modification is available (see Chapter 7.1 Insertions larger than 80 bp).

If there is no prior knowledge, the strategies used for detection of unknown GMOs may be applied to detect the large modifications resulting from ZFN-3. Identification will however not be possible without prior knowledge.

Oligonucleotide directed mutagenesis (ODM)

Mutations that are the result of ODM can be detected by PCR-based methods as long as certain information on the nucleotides in the vicinity of the mutation is known. This is necessary to be able to design primers. Without such information, the mutation cannot even be detected.

In any case, methods allowing the detection of mutations do not permit identification of ODM products.

It is not possible to distinguish, at the molecular level, organisms developed through ODM from organisms bearing the same mutation obtained through other mutation techniques (chemical or radiation mutagenesis). It is also not possible to differentiate ODM products from spontaneous mutations or single nucleotide polymorphism mutations (see Chapter 7.1 Modification of a few nucleotides).

Cisgenesis and intragenesis

Cisgenic/intragenic plants harbour genes that were derived from within the gene pool of the same species.

Cisgenic/intragenic plants can be detected and identified as such when the event is known beforehand, i.e. when adequate information about the cisgenesis/intragenesis modification is made available (see Chapter 7.1 Insertions larger than 80 bp). Event-specific primers can be developed to create a detection and identification method.

30 A new document from the ENGL on "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials" is under preparation and is expected to be published in 2011. This upcoming ENGL publication will provide further detailed information on the challenges raised by the detection of "unknown" GMOs, which may be relevant to the ones raised in the present report under the scenario "Without prior knowledge".

In the case of unknown alterations, sequencing (genome or transcriptome) could in theory support the detection of cisgenic/intragenic plants but the method has not yet been validated for this purpose. Therefore it can be concluded that without prior knowledge, the detection and the identification of cisgenic and intragenic plants is not currently feasible.

RNA-dependent DNA methylation (RdDM)

Specific gene silencing is obtained through DNA methylation and/or histone methylation in the chromatin but the DNA sequence itself is not modified.

Since it is very difficult to differentiate between methylation occurring naturally and methylation through the deliberate use of a technique like RdDM, it can be concluded that identification of RdDM products is not possible, even with prior knowledge.

Grafting (on GM rootstock)

Grafting of a non-GM scion onto a GM rootstock is the case on which the NTTF focused.

As the DNA sequence of the non-GM scion is not modified, detection and identification of the GM rootstock on the basis of the harvested product (part of the non-GM scion) is not currently possible and is very unlikely to be developed in the near future.

Reverse breeding

The end-products of reverse breeding are free of genetic modification-related DNA sequences because the homozygous parental lines are produced from double-haploid plants which are screened for the absence of RNAi construct during the breeding process.

It is therefore not possible to distinguish products resulting from the use of the reverse breeding technique from products resulting from conventional breeding. Identification of products resulting from the use of reverse breeding technique is therefore not possible.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

If the constructs introduced into plants by agro-infiltration are not replicated and/or integrated, their presence is transient and can be detected only in the agro-infiltrated plant itself. These DNA fragments will not be transferred to the next generation so they cannot be detected or identified in the progeny plant and the products derived thereof. Detection and identification of products from agro-infiltration or from agro-inoculation is therefore not possible.

Detection and identification of agro-infiltrated plants and progeny plants that contain stably inserted fragments is possible with the same methodologies that are currently developed and used for GMO detection, which also implies that adequate information needs to be available.

In the case of floral dip, the aim is to select for stable integration into the germline, leading to a genetically modified plant, which means that detection and identification are possible with the methods currently available for GMO detection (PCR), and also implies that adequate information needs to be available.

If no prior information is available, identification will not be possible under any circumstances.

Conclusions on identification of new plant breeding techniques:

The following conclusions were agreed by the NTTF (a summary table is included at the end of Annex 16):

It is *not possible to identify* products from the following new plant breeding techniques (mainly because they cannot be differentiated from products obtained with conventional breeding methods, with other mutation techniques (chemical or radiation mutagenesis) or through natural mutations):

1. Zinc finger nuclease technology 1 and 2
2. Oligonucleotide directed mutagenesis (ODM)
3. RNA-dependent DNA methylation (RdDM)
4. Grafting on a GM rootstock
5. Reverse breeding
6. Agro-infiltration (agro-infiltration and agro-inoculation)

It is possible to *identify products* from the following new plant breeding techniques, provided some prior information is available (about the DNA sequence introduced by the genetic modification and the neighbouring genomic DNA sequence):

1. Zinc finger nuclease technology 3
2. Cisgenesis and intragenesis
3. Agro-infiltration (floral dip)

Without any prior knowledge about the genetic modification introduced by a specific new plant breeding technique, it is not possible to identify products from this new technique.

8 ADDITIONAL RESEARCH NEEDS AND NEW TECHNIQUES IDENTIFIED

8.1 Further needs for technical research

The JRC project aims to provide information on the state-of-the-art of the research into and the adoption of new plant breeding techniques for the policy maker. After collecting available data and carrying out evaluations in specified fields, we conclude by focusing on the identification of additional research needs, not only for further development of the technologies but also from the point of view of providing a solid basis for decision making.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

A protocol for the delivery of the genes coding for the ZFNs into the plant cell and for the regeneration of plants from tissue cultures has to be developed for each crop on a case-by-case basis. Research is underway to deliver the ZFNs as proteins.

Currently ZFNs for approximately half of the 64 nucleotide triplets are available. ZFN libraries are being updated to improve genome coverage. It is also necessary to improve the specificity and efficiency of ZFNs. ZFNs are subject to an extensive selection and validation process before being commercialised. In parallel smart approaches for selection of the mutated plants have to be developed.

Further investigations have to be carried out to clarify whether genes coding for ZFNs are only expressed transiently or if they are integrated in the genome.

Furthermore, the extent to which the ZFN technique is applicable for the induction of mutations in all alleles of polyploidy crops or of paralogous genes or of cluster genes is still to be determined.

Oligonucleotide directed mutagenesis (ODM)

ODM has to be applied to protoplasts. This limits its application to certain crops and expertise for the production and regeneration of protoplasts has to be acquired. To achieve higher mutation efficiency, the design of the oligonucleotides has to be improved. Furthermore, methods for efficient screening of the mutated plants have to be developed.

Cisgenesis and intragenesis

Cisgenesis/intragenesis takes advantage of the experience gained in the use of transgenesis, a technology that in principle applies the same plant transformation methods. However, some problems related specifically to cisgenesis/intragenesis still have to be addressed, such as the search for and isolation of suitable genes within the gene pool of the crops, investigation of the functioning of plant-derived promoters or the development of marker-free approaches.

RNA-dependent DNA methylation (RdDM)

The applicability of RdDM has to be investigated on more crop plants and the durability of the gene silencing in particular has to be investigated and improved. Furthermore the design of the transgene encoding dsRNA needs to be improved. Methylation is restricted to the region of sequence homology with the dsRNA. Therefore, it is necessary to investigate further the functioning of the promoters and especially to study which sequences are relevant for the regulation of gene expression.

Grafting (on GM rootstock)

Grafting on GM rootstock combines two breeding techniques with a long history of use: grafting and genetic transformation. Therefore, the technique is well developed. However, while the influence of different rootstocks on the physical appearance of the scions is known, knowledge of the movement of molecules from the rootstock to the scion and their influence on gene expression in the scion need to be investigated further.

Reverse breeding

Reverse breeding is a very young technique and therefore research is still required to overcome technical problems and to fully exploit its potential. For example, research is being carried out to test alternatives to transformation for obtaining the suppression of recombination, like VIGS (Virus Induced Gene Silencing), graft transmission of silencing molecules, knock-out mutations or the use of chemicals that repress crossover. Additional research is needed to improve the efficiency of DH formation.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)

The technique is well developed. However, to date it is only applied in a small number of plant species and tissues. Research into in the possible expansion of its applicability might be of interest in the future.

Although only transient and local gene expression is intended, spreading and integration of *Agrobacterium* and integration of the T-DNA cannot be excluded. Further research is therefore required, including the testing for the presence of *Agrobacterium* and for the integration of T-DNA.

8.2 Additional new plant breeding techniques

The NTWG and the current JRC project focus on a list of only eight techniques, seven of which are relevant for plant breeding. During the JRC project we found that the commercial adoption of a further new plant breeding technique, the meganuclease technique, is relatively advanced (phase I). Like ZFNs, meganucleases can be used for site-specific mutagenesis or for targeted gene insertion by homologous recombination. This suggests that the meganuclease technique should be considered in the discussion on the classification of new plant breeding techniques under the GMO legislation.

In the survey of plant breeding companies, some further new plant breeding techniques were mentioned, but with lower adoption rates (just one company per technique). These technologies concerned the delivery of DNA modifying enzymes (e.g. ZFNs or homing nucleases) into the plant cells or involved transgenic inducer construct-driven breeding tools[31].

31 For the definition refer to Annex 9.

Annex 1: Legal background

Harmonised EU GMO legislation goes back to the year 1990, when Directive 90/220/EEC, on the deliberate release of GMOs into the environment[32], and Directive 90/219/EEC, on the contained use of genetically modified micro organisms (GMMs)[33], came into force.

The legislation has since been revised and up dated. Directive 90/220/EEC has been replaced by Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms[34]. Directive 90/219/EEC was amended by Directive 98/81/EC[35] and replaced by Directive 2009/41/EC[36] on the contained use of genetically modified micro-organisms. Additional legislation was introduced in 2003 to regulate genetically modified food and feed[37].

Because of difficulties concerning the implementation of the legislation an evaluation of the EU legislative framework was launched in 2009. Two consortia carried out the evaluation of the EU legislative framework in the field of GM food and feed and of the EU legislative framework in the field of cultivation of GMOs under Directive 2001/18/EC, respectively.

32 Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms - OJ L 117, 8.5.1990, p. 15–27

33 Council Directive 90/219/EEC of 23 April 1990 on the contained use of genetically modified micro-organisms - OJ L 117, 8.5.1990, p. 1-14

34 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 - OJ L 106, 17.4.2001, p. 1–39

35 Council Directive 98/81/EC of 26 October 1998 amending Directive 90/219/EEC on the contained use of genetically modified micro-organisms - OJ L 330, 5.12.1998, p. 13–31

36 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 - OJ L 125, 21.5.2009, p. 75–97

37 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) - OJ L 268, 18.10.2003, p. 1–23

Annex 2: GMO definition

Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms[38]

Article 2

Definitions

For the purposes of this Directive:

(1) "organism" means any biological entity capable of replication or of transferring genetic material;

(2) "genetically modified organism (GMO)" means 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.

Within the terms of this definition:

(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1;

(b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification.

Article 3

Exemptions

1. This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B.

ANNEX I A

TECHNIQUES REFERRED TO IN ARTICLE 2(2)

PART 1

Techniques of genetic modification referred to in Article 2(2)(a) are inter alia:

(1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation;

(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;

(3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

38 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 - OJ L 106, 17.4.2001, p. 1–39

PART 2

Techniques referred to in Article 2(2)(b) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms made by techniques/methods other than those excluded by Annex I B:

(1) *in vitro* fertilisation,

(2) natural processes such as: conjugation, transduction, transformation,

(3) polyploidy induction.

ANNEX I B

TECHNIQUES REFERRED TO IN ARTICLE 3

Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:

(1) mutagenesis,

(2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.

Annex 3: Literature search - Methodology

The bibliographic database ISI Web of science was employed for the literature search on new plant breeding techniques since it is considered as one of the most comprehensive literature databases[39].

The techniques for which we searched are the techniques listed by the NTWG (see Chapter 2), with the exception of synthetic genomics. The latter was excluded due to the absence of publications related to the application of synthetic genomics for plant breeding.

The literature search was performed through search keywords, specifically chosen for each of the seven techniques. 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 the new techniques is presented below. 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.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
- "zinc finger nucleas*" **AND** plant*
- ZFN **AND** plant*

Oligonucleotide directed mutagenesis (ODM)
- "oligonucleotide directed mutagenesis" **AND** plant*
- "chimeric oligonucleotid*" **AND** plant*
- "chimeric RNA/DNA oligonucleotid*" **AND** plant*
- chimeraplasty **AND** plant*
- "site-directed mutagenesis" **AND** oligonucleotid* **AND** plant*
- "gene targeting" **AND** oligonucleotid* **AND** plant*

Cisgenesis and Intragenesis
- cisgen*
- intragenesis
- "all native DNA transformation"
- "native DNA" **AND** plant*

RNA-dependent DNA methylation (RdDM)
- "RNA dependent DNA methyl*" **AND** plant*
- "RNA directed DNA methyl*" **AND** plant*
- RdDM **AND** plant*
- "transcriptional gene silencing" **AND** "double stranded RNA" **AND** methyl* **AND** plant*
- "transcriptional gene silencing" **AND** dsRNA **AND** methyl* **AND** plant*
- "RNA mediated transcriptional gene silencing" **AND** plant*

39 The literature search was finalised in April 2010. Therefore results include all scientific publications on new plant breeding techniques published until that date.

Grafting (on GM rootstock)
- graft* **AND** "transg* rootstock*"
- graft* **AND** "transform* rootstock*"
- graft* **AND** "GM rootstock*"
- graft* AND "WT scion*"
- graft* AND "wild type scion*"

Reverse Breeding
- "reverse breeding"
- "crossover control" **AND** breeding **AND** plant*

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)
- agroinfiltr*
- agroinocul*
- agroinfect*

Literature results for floral dip were not analysed further as plants derived from this technique do not differ from GM plants obtained by other transformation methods and therefore the technique is not considered as relevant for discussion.

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 lose information, since the general number of publications about the seven new plant breeding techniques is quite low (23 on average per technique).

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;
- Private, public or mixed institutions (based on the address of the author(s)).

Research papers additionally were categorised according to:
- Plant on which the technique was used;
- Trait obtained through the application of the technique;
- For ZFN technology: use of ZFN-1, -2 or -3 (see Chapter 3.1).

Data for the seven 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.

ANNEX 4: LITERATURE SEARCH - DETAILED RESULTS

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

Figures 6, 7, 8 and table 5 show the specific results obtained for ZFN technology. Through the keyword analysis, as explained in Annex 3, nine research papers and 11 review papers[40] have been identified on the use of ZFN technology in plant breeding. As mentioned in Chapter 4.1, the USA produced the highest number of publications (Figure 6). As illustrated in Table 5 and in Figure 8, all three ZFN techniques (ZFN-1, -2 and -3, see Chapter 3.1) were identified in research papers. Details on plants used and traits obtained are illustrated in Table 5 and Figure 7.

Figure 6: Geographical distribution of scientific publications (both review and research papers) on ZFN technology.

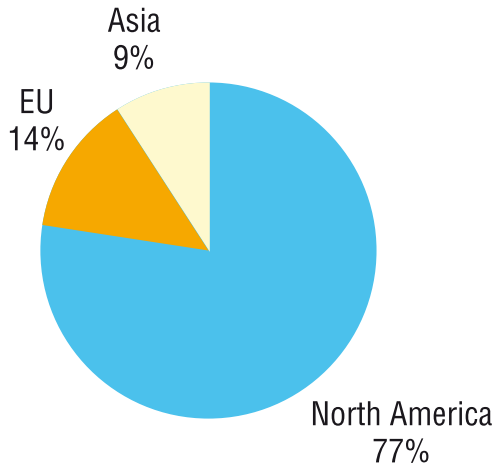
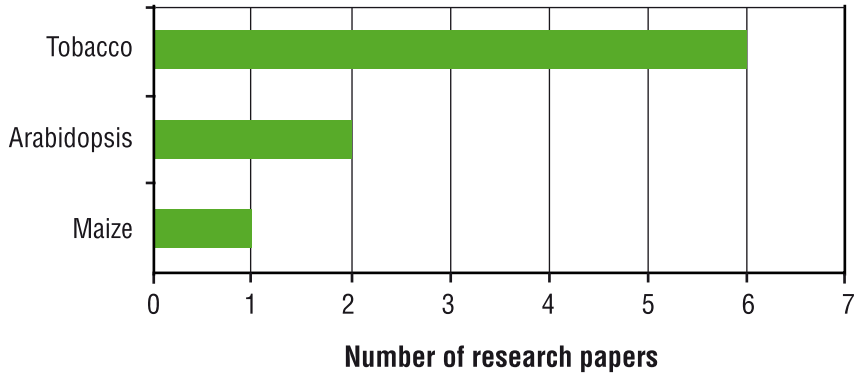


Figure 7: Plant species used for ZFN technology, according to data from research papers.



40 List of review papers on ZFN technology: Alonso and Ecker (2006), Durai et al. (2005), Jander and Barth (2007), Kumar et al. (2006), Li et al. (2007), Moeller and Wang (2008), Puchta and Hohn (2005), Saika and Toki (2009), Tzfira and White (2005), Wright et al. (2006), Wu et al. (2007)

Figure 8: Distribution of research papers on ZFN-1, -2 and -3 technology.

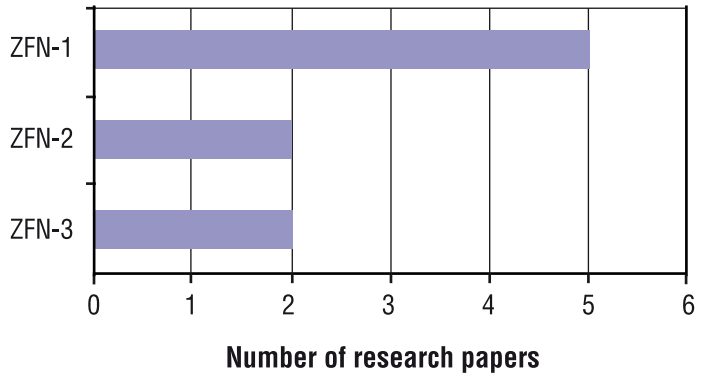


Table 5: List of research papers on ZFN technology and identification of country of authors, plants employed, traits obtained, genes involved and type of ZFN technique used.

Paper	Country	Plant	Trait	Gene	ZFN-1, -2, -3
CAI et al (2009)	USA	Tobacco	Herbicide tolerance	PAT gene (phosphinothricin phosphotransferase)	ZFN-3
DE PATER et al (2009)	NL	Arabidopsis	Mutation of reporter gene	reporter genes: GFP/GUS	ZFN-2
LLOYD et al (2005)	USA	Arabidopsis	Mutation of model construct	construct with EcoRI site	ZFN-1
MAEDER et al (2008)	USA	Tobacco	Herbicide tolerance	ALS genes (acetolactate synthase)	ZFN-1
SHUKLA et al (2009)	USA	Maize	Herbicide tolerance	PAT gene (phosphinothricin phosphotransferase)	ZFN-3
TOVKACH et al (2009)	USA	Tobacco	Cleavage of reporter gene	reporter gene: GUS	ZFN-1
TOWNSEND et al (2009)	USA	Tobacco	Herbicide tolerance	ALS gene (acetolactate synthase)	ZFN-1
WRIGHT et al (2005)	USA	Tobacco	Mutation of reporter gene	reporter gene: GUS:NPTII	ZFN-2
ZEEVI et al (2008)	USA	Tobacco	Cleavage of model gene	FtsH2 gene (ATP-dep. chloroplast protease)	ZFN-1

Oligonucleotide directed mutagenesis (ODM)

For ODM, ten research papers and 15 review papers[41] were found in literature. Figure 9 shows that institutions both in the EU and in North American published papers on the use of this technique in plants. Details on plants used and traits obtained are illustrated in Table 6 and Figure 10.

Figure 9: Geographical distribution of scientific publications (both review and research papers) on ODM.

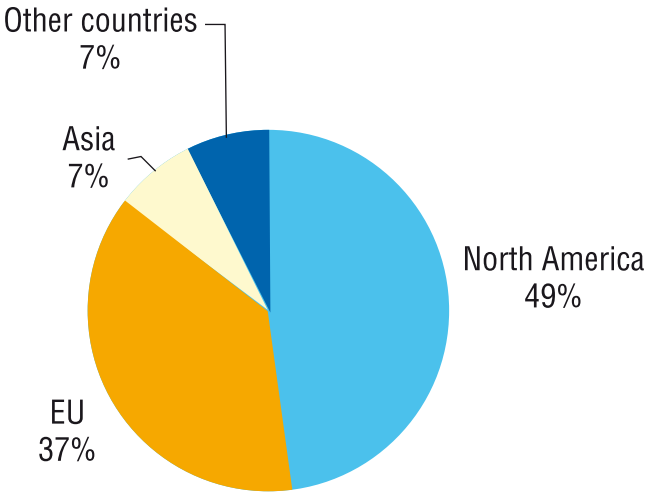
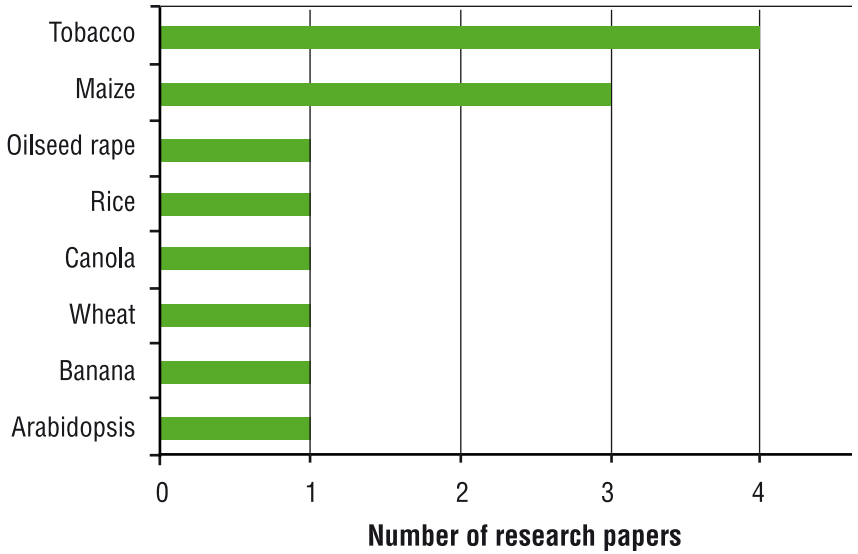


Figure 10: Plant species used for ODM, according to data from research papers.



41 List of review papers on ODM: BAC (2007), Belzile (2002), Breyer et al. (2009), Britt and May (2003), Hohn and Puchta (1999), Iida and Terada (2005), Kumar and Fladung (2001), Oh and May (2001), Puchta (2002), Puchta (2003), Reiss (2003), Rice et al. (2001), Shewry and Jones (2005), Stewart et al. (2000), Tranel and Wright (2002)

Table 6: List of research papers on ODM and identification of country of authors, plants employed, traits obtained and genes involved.

Research paper	Country	Plant	Trait	Gene
BEETHAM et al (1999)	USA	Tobacco	Herbicide tolerance	ALS gene (acetolactate synthase)
DONG et al (2006)	AU, US	Wheat	Green fluorescence	marker gene: GFP
GAMPER et al (2000)	USA	Canola	Antibiotic resistance	marker gene: kanamycine resistance
KMIEC et al (2001)	USA	Arabidopsis	Antibiotic resistance	marker gene: kanamycine resistance
KOCHEVENKO & WILLMITZER (2003)	DE	Tobacco	Herbicide tolerance	ALS gene (acetolactate synthase)
OKUZAKI & TORIYAMA (2004)	JP	Rice	Herbicide tolerance	ALS gene (acetolactate synthase)
RICE et al (2000)	USA	Maize, Banana, Tobacco	Antibiotic resistance	marker genes: kanamycine, tetracycline resistance
RUITER et al (2003)	BE	Tobacco, Oilseed rape	Herbicide tolerance	ALS gene (acetolactate synthase) bar, bar+egfp
ZHU et al (1999)	USA	Maize	Herbicide tolerance	AHAS (acetohydroxyacid synthase)
ZHU et al (2000)	USA	Maize	Herbicide tolerance	AHAS (acetohydroxyacid synthase)

Cisgenesis and intragenesis

Ten research papers and 26 review papers[42] were identified for cisgenesis and intragenesis. Figure 11 illustrates the leading role of EU countries, especially of the Netherlands. Details on plants used and traits obtained are illustrated in Table 7 and Figure 12.

Figure 11: Geographical distribution of scientific publications (both review and research papers) on cisgenesis and intragenesis.

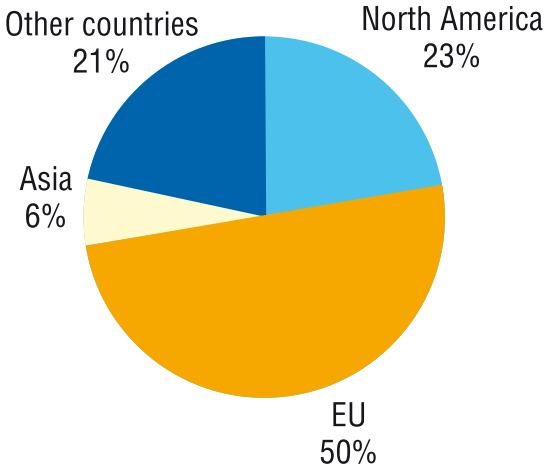
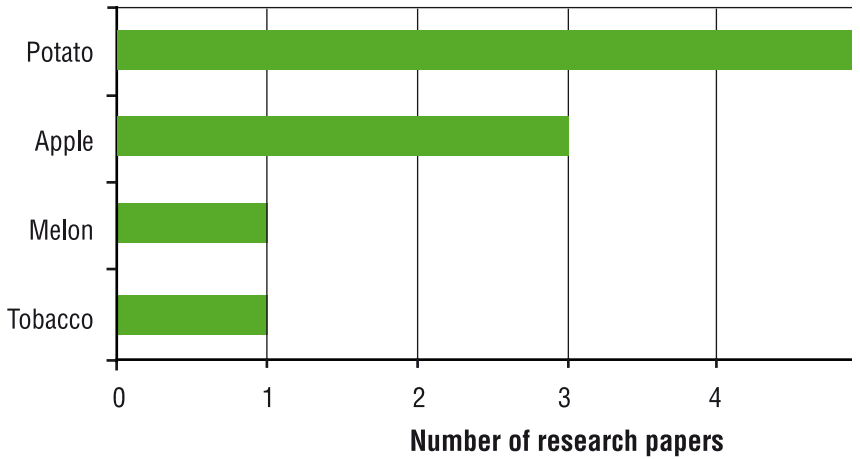


Figure 12: Plant species used for cisgenesis and intragenesis, according to data from research papers.



42 List of review papers on cisgenesis/intragenesis: Akhond and Machray (2009), COGEM (2006a), Conner et al. (2007), Haverkort et al. (2008), Jacobsen and Nataraja (2008), Jacobsen and Schouten (2008), Jacobsen and Schouten (2007), Jacobsen and Schouten (2009), Jacobsen et al. (2009), Kok et al. (2008), Lammerts Van Bueren et al. (2007), Myskja (2006), Nielsen (2003), Park et al. (2009b), Rommens (2004), Rommens (2007), Rommens (2008), Rommens (2010), Rommens et al. (2007), Russell and Sparrow (2008), Schouten and Jacobsen (2007), Schouten and Jacobsen (2008), Schouten et al. (2006a), Schouten et al. (2006b), Schouten et al. (2009), Schubert and Williams (2006).

Table 7: List of research papers on cisgenesis and intragenesis and identification of country of authors, plants employed, traits obtained and genes involved

Paper	Country	Plant	Trait	Gene
BELFANTI et al (2004)	IT, CH	Apple	fungal resistance	Vf gene
BENJAMIN et al (2009)	IL	Melon	fungal resistance	At1/At2 - glyoxylate aminotransferase
KUHL et al (2007)	USA	Potato	fungal resistance	RB gene
PARK et al (2009a)	UK, KR	Potato	fungal resistance	Rpi genes
ROMMENS et al (2004)	USA	Potato	black spot bruise tolerance	PPO gene
ROMMENS et al (2005)	USA	Tobacco	Insertion of model genes	model genes: KpnI, SpeI sites
ROMMENS et al (2006)	USA	Potato	lower acrylamide levels	Ppo (polyhenol oxidase) and PhL (phosphorylase-L)
ROMMENS et al (2008)	USA	Potato	lower acrylamide levels	asparagine synthetase genes (StAs1 and StAs2)
SILFVERBERG-DILWORTH et al (2005)	IT, CH	Apple	fungal resistance	HcrVf2 gene
SZANKOWSKI et al (2009)	DE, CH, IT, BR	Apple	fungal resistance	HcrVf2 gene

RNA-dependent DNA methylation (RdDM)

Identified publications on RdDM were divided into two categories: i) publications describing the natural phenomenon of RdDM in plants and all discoveries related to this phenomenon and ii) publications investigating the induction of RdDM in plants in order to obtain changes in gene expression. Seventeen research papers and 14 review papers[43] belonging to the second category were identified. As illustrated in Figure 13, most of them were published by authors from the EU. Details on plants used and traits obtained are illustrated in Table 8 and Figure 14.

Figure 13: Geographical distribution of scientific publications (both review and research papers) on RdDM.

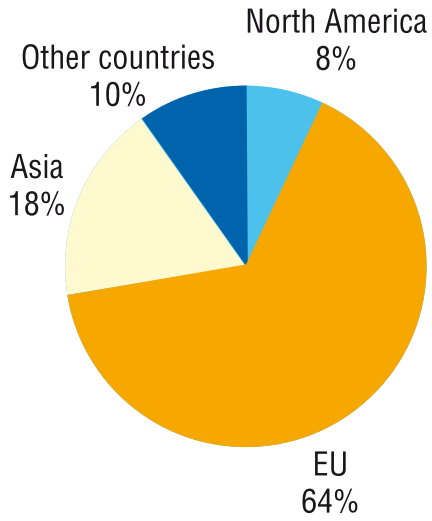
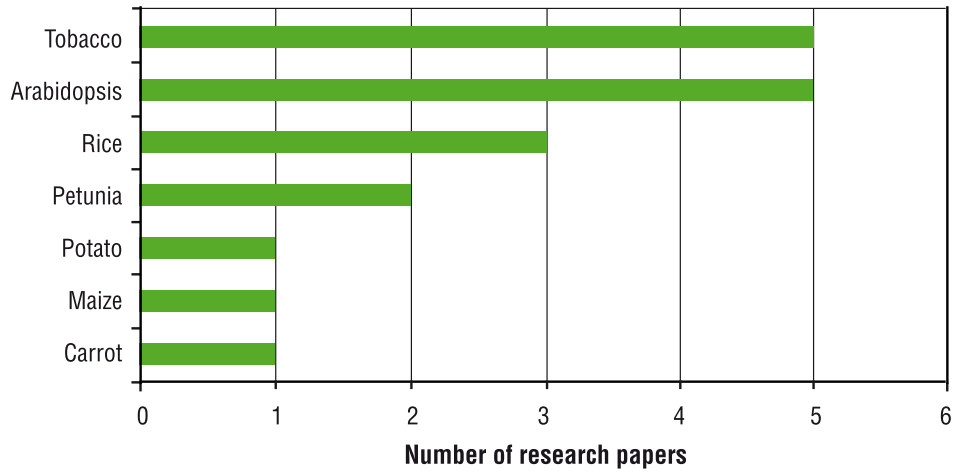


Figure 14: Plant species used for RdDM, according to data from research papers.



43 List of review papers on RdDM: Chen (2010), Chinnusamy and Zhu (2009), Eamens et al. (2008), Huettel et al. (2007), Lavrov and Kibanov (2007), Matzke et al. (2004), Matzke et al. (2001), Muskens et al. (2000), Pickford and Cogoni (2003), Shiba and Takayania (2007), Vaucheret and Fagard (2001), Verdel et al. (2009), Wang and Waterhouse (2002), Wassenegger (2000)

Table 8: List of research papers on RdDM and identification of country of authors, plants employed, traits obtained and genes involved.

Paper	Country	Plant	Trait	Gene
AUFSATZ et al (2002a)	AT	Arabidopsis	silencing of marker gene	NOSpro-NPTII
AUFSATZ et al (2002b)	AT	Arabidopsis	silencing of marker gene	NOSpro-NPTII
CIGAN et al (2005)	USA	Maize	male-sterility	Ms45 promoter
DALAKOURAS et al (2009)	DE	Tobacco	silencing of marker gene	GFP
DAXINGER et al (2009)	AT	Arabidopsis	silencing of marker gene	GFP
FISCHER et al (2008)	DE	Arabidopsis	silencing of marker gene	NOSpro-NPTII
FU et al (2000)	UK	Rice	silencing of model genes	heterologous transgenes bar, hpt, gusA (models)
HEILERSIG et al (2006)	NL	Potato	modified starch content	GBSSI promoter (granule bound starch synthase I)
KAPOOR et al (2005)	JP	Petunia	silencing of endogenous genes	pMADS3 (homeotic gene)
KUNZ et al (2003)	AT, FR, CZ, TH, KR, AR	Tobacco	silencing of marker gene	NOSpro-NPTII
LUNEROVA-BEDRICHOVA et al (2008)	CZ, BE	Tobacco	silencing of marker gene	NPTII
METTE et al (1999)	AT	Tobacco	silencing of marker gene	NOSpro-NPTII
METTE et al (2000)	AT	Tobacco, Arabidopsis	silencing of marker gene	NOSpro-NPTII
MIKI & SHIMAMOTO (2008)	JP	Rice	silencing of endogenous genes	OsRac genes
OKANO et al (2008)	JP	Rice	silencing of model genes	GFP, OsRac, Cen
SHIBUKAWA et al (2009)	JP	Carrot (cells)	analysis of embryogenesis transcription factor	C-LEC1 (Carrot-Leafy Cotyledon 1)
SIJEN et al (2001)	NL	Petunia	reduced flower pigmentation	promoter gene chalcone synthaseA - chsA

Grafting (on GM rootstock)

Regarding grafting, 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 improvement. Twenty nine research papers and two review papers[44] belonging to the second category were identified. As illustrated in Figure 15, most publications have been produced by authors from the EU. Details on plants used and traits obtained are illustrated in Table 9 and Figure 16.

Figure 15: Geographical distribution of scientific publications (both review and research papers) on grafting on GM rootstock.

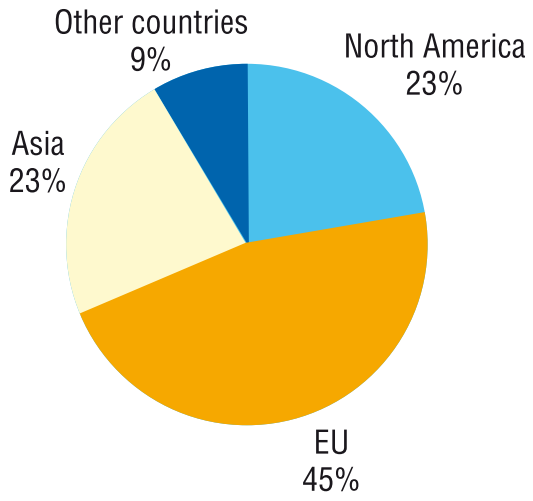
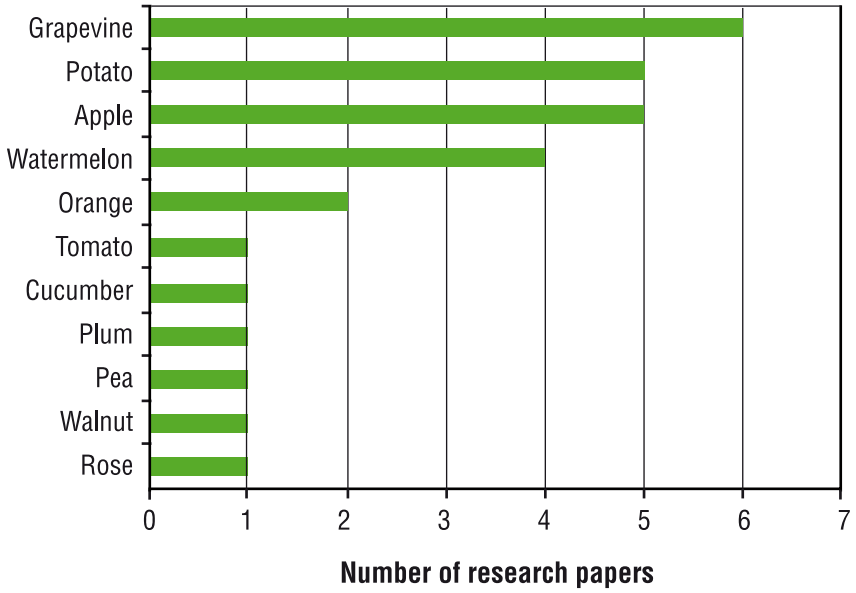


Figure 16: Plant species used for grafting on GM rootstock, according to data from research papers.



44 List of review papers on grafting on GM rootstock: Lough and Lucas (2006), van der Salm et al. (1996).

Table 9: List of research papers on grafting on GM rootstock and identification of country of authors, plants employed, traits obtained and genes involved.

Paper	Country	Plant	Trait	Gene
AGUERO et al (2005)	USA	Grapevine	bacteria and fungal resistance	pPGIP (polygalacturunase-inhibiting protein)
CONRATH et al (2003)	UK, DE	Potato	fungal resistance	transporter AATP1
COUTOS-THEVENOT et al (2001)	FR, DE	Grapevine	fungal resistance	gene: Vst1 (Vitis stilbene synthase 1)
DERRICK & BARKER (1997)	UK	Potato	virus resistance	PLRV coat protein
GAL-ON et al (2005)	IL, KR	Cucumber	virus resistance	replicase gene of CFMMV
GAMBINO et al (2005)	IT, AT	Grapevine	virus resistance	GFLV CP
GEIER et al (2008)	DE	Grapevine	rooting ability	rolB gene
HAN et al (2009)	USA, KR	Watermelon	robust growth	Ca2+/H+ exchanger sCAX2B
KIM et al (2008)	KR	Watermelon	virus resistance	CGMMV-CP gene (virus coat protein)
KRASTANOVA et al (2010)	USA	Grapevine	bacterial resistance	virE2
KUHN et al (1996)	DE, UK	Potato	inhibition of transporter = accumulation of carbohydrates (lower tuber yield)	sucrose transporter SUT1
LAMBERT & TEPFER (1991)	FR	Apple	rooting ability	rolC
MACKENZIE et al (1991)	CA	Potato	virus resistance	Potato virus (PTV) coat protein
MAKI-VALKAMA et al (2000)	SE, FI	Potato	virus resistance	P1 sequence of potato virus Y (PVY)
MCGURL et al (1994)	USA	Tomato	defense against herbivorous insects	proteinase inhibitors I and II
MITANI et al (2006)	JP	Orange	fungal resistance	RRC2 (rice chitinase)
MOLINARI et al (2004)	BR	Orange	improved osmotic control	D1-pyrroline-5-carboxylate synthetase mutant gene (p5cs)
NAGEL et al (2010)	USA	Plum	fungal/nematodes resistance	GAFP-I (Gastrodia antifungal protein)
PARK et al (2005)	KR	Watermelon	virus resistance	CGMMV-CP gene (virus coat protein)
VAHDATI et al (2002)	USA	Walnut	rooting ability	rolA,B,C
VAN DEN BOOGAART et al (2004)	UK	Pea	virus resistance	PSbMV sequence
VAN DER SALM et al (1998)	NL	Rose	rooting ability	rolA,B,C
VERNOOIJ et al (1994)	USA, CH	Tobacco	bacterial resistance	salicylic acid degrading enzymes
VIGNE et al (2004)	FR	Grapevine	virus resistance	GFLV coat protein
WELANDER et al (1998)	SE	Apple	rooting ability	rolB gene
XU et al (2009)	CN	Apple	insertion of reporter gene	reporter gene: GUS
YOUK et al (2009)	KR	Watermelon	virus resistance	CGMMV-CP gene (virus capsid protein)
ZHU & WELANDER (1999)	SE	Apple	rooting ability	rolA,B,C
ZHU et al (2001)	SE	Apple	rooting ability	rolB gene

Reverse breeding

Only three review papers[45] on the technique of reverse breeding were identified: two of them published by a Dutch public institute and the third one by a joint collaboration between academy and industry, involving the Netherlands, Austria, the USA and China.

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

154 publications were identified on agro-infiltration "sensu stricto", 236 on the use of agro-inoculation and 80 on the use of floral dip, 20 of which describe transformations for plant improvement. As stated in Chapter 4.1, literature results for floral dip were not analysed further as plants derived from this technique do not differ from GM plants obtained by other transformation methods.

Most of the publications describe the use of agro-infiltration or agro-inoculation for research purposes. Only publications relevant for plant breeding have been selected: 26 publications (25 research papers and 1 review[46]) on the use of agro-infiltration for the production of recombinant proteins in plants and ten research papers on the use of agro-infiltration for screening of pest resistance in plants.

Figure 17 shows that the EU and North America (representing mostly the USA) have published a similar number of scientific papers. Details on plants used and traits obtained are illustrated in Table 10 for the use of agro-infiltration for production of recombinant proteins and in Table 11 for the use of agro-infiltration for the screening of pest resistance. In Figure 18 plants employed in both groups of articles are illustrated.

45 List of review papers on reverse breeding: Dirks et al. (2009), Lammerts Van Bueren et al. (2007), Wijnker and de Jong (2008)
46 Fischer et al. (1999)

Figure 17: Geographical distribution of scientific publications (both review and research papers) on agro-infiltration.

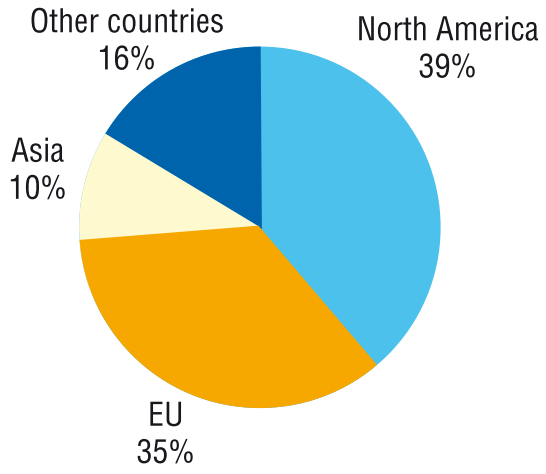


Figure 18: Plant species used for agro-infiltration, according to data from research papers.

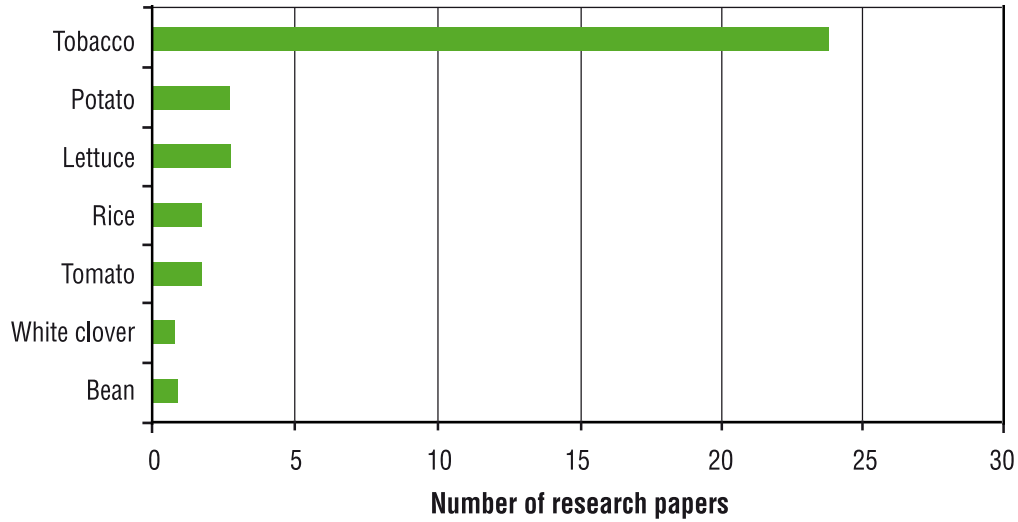


Table 10: List of research papers on agro-infiltration used for the production of recombinant proteins and identification of country of authors, plants employed, proteins produced and genes involved.

Paper	Country	Plant	Protein produced	Gene
D'Aoust et al (2008)	CA, FR	N.benthamiana	influenza vaccine	haemagglutinin (HA)
Ferraro et al (2008)	AR	tobacco	anti-Toxoplasma vaccine	Gra4 antigen
Fujiki et al (2008)	USA	N.benthamiana	Human growth hormone	hGH
Gomez et al (2009)	AR	N.benthamiana	New Castle Disease vaccine	HN glycoprotein of NDV
Huang et al (2009)	USA	N.benthamiana	hepatitis B and Norwalk virus vaccines	hepatitis B core antigen (HBc) and Norwalk virus capsid protein (NVCP)

Paper	Country	Plant	Protein produced	Gene
Hull et al (2005)	USA	N.benthamiana	diabetes vaccine and tetanus antibody	diabetes associated antigen, IA-2ic and anti-tetanus antibody 9F12
Joh et al (2005)	USA	lettuce	reporter gene expression	reporter gene: GUS
Joh & VanderGheynst (2006)	USA	lettuce	reporter gene expression	reporter gene: GUS
Lee et al (2001)	CA	white clover	vaccines against bovine pneumonia pasteurellosis	A1 leukotoxin (Lkt)
Li et al (2006)	CN	lettuce, tobacco	vaccine against SARS-CoV	spike (S) protein of SARS-CoV
Lombardi et al (2009)	IT	N.benthamiana	HIV vaccine	HIV antigen Nef
Mett et al (2007)	USA	N.benthamiana	diabetes vaccine	human IA-2 (IA-2ic)
Meyers et al (2008)	ZA	Nicotiana	HIV vaccine	HIV-1 Pr55Gag
Plesha et al (2009)	USA	N.benthamiana	therapeutic protein for AAT deficiency	recombinant alpha-1-antitrypsin (rAAT)
Pogue et al (2010)	USA	Nicotiana	aprotinin and monoclonal antibody against HIV	r-aprotinin gene and Anti-(α CCR5 mAbs
Rance et al (2002)	FR	tobacco	human lactoferrin	hLf
Rodriguez et al (2005)	CU	tobacco	antibody ag. the Epidermal Growth Factor receptor (EGF-R): cancer therapy	TheraCIMR
Sainsbury et al (2008)	UK	N.benthamiana	antibodies against HIV and Hepatitis B	human anti-HIV antibody 2G12 and Hepatitis B core antigen (HBcAg)
Sainsbury et al (2008)	UK, CA	N.benthamiana	blood typing antibody	immunoglobulin G (IgG) C5-1
Srinivas et al (2008)	IN	tomato	hepatitis B vaccine	Hepatitis B surface antigen (HBsAg)
Steel et al (2010)	UK, PE	N.benthamiana	criniviruses antibodies (plant virus)	CYSDV coat protein
Sudarshana et al (2006)	USA	N.benthamiana	therapeutic protein for AAT deficiency	recombinant alpha-1-antitrypsin (rAAT)
Triques et al (2008)	FR, USA	N.benthamiana	mismatch-specific endonuclease	ENDO1
Vezina et al (2009)	CA, FR	N.benthamiana	antibody, a suppressor of silencing and a chimaeric human β1,4-galactosyltransferase	C5-1 IgG, HcPro and chimaeric human GalT
Zelada et al (2006)	AR	N.benthamiana	vaccine against Tubercolosis	ESAT-6 protein

Table 11: List of research papers on agro-infiltration used for screening of pest resistance in plants and identification of country of authors, plants employed, resistance identified and genes involved.

Paper	Country	Plant	Trait verified	Gene
Armstrong et al (2005)	NL, USA, UK	N. benthamiana	resistance to P.infestans	Avr3a and R3a
Bendahmane et al (2000)	UK, PE	N. tabacum	potato virus resistance	Rx1 and Rx2
Cruz et al (1999)	PH, UK	rice	Resistance to Rice Tungro Bacilliform Virus	RTBV
Erickson et al (1999)	USA	tobacco	Tobacco mosaic virus (TMV)	TMV helicase fragment (p50)
Garrido-Ramirez et al (2000)	USA	common bean (Phaseolus vulgaris)	Infectivity of Bean golden yellow mosaic virus	BGYMV-MX
Mestre et al (2003)	UK	potato	potato virus resistance	PVY NIaPro
Tripathi & Varma (2003)	IN	Lycopersicon species (tomato)	resistance to Tomato leaf curl geminivirus	ToLCV
Vleeshouwers et al (2006)	USA, NL	Solanum plants	resistance to Phytophthora infestans	elicitins INF1, INF2A and INF2B
Vleeshouwers et al (2008)	NL, USA, UK	Solanum stoloniferum	resistance to P.infestans	pGR106-Ipi0
Zenna et al (2006)	PH, AU	rice	Resistance to Rice Tungro Bacilliform Virus	RTBV

ANNEX 5: PATENT SEARCH - METHODOLOGY

Three public patent databases were explored for the search: WIPO (World Intellectual Property Organization), EPO (European Patent Office) and USPTO (United States Patent and Trademark Office)[47]. Results of the search include both patent applications and issued patents.

As for the literature search, we searched for the techniques listed by the NTWG (see Chapter 2), with the exception of synthetic genomics. The latter was excluded due to the absence of patents related to the application of synthetic genomics for plant breeding.

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 prepare 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 literature search for the new techniques is presented below. 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.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
- "zinc finger" **AND** nuclease* **AND** plant **AND** break
- "zinc finger" **AND** NHEJ

Oligonucleotide directed mutagenesis (ODM)
- "chimeric oligonucleotide*" **AND** plant

Cisgenesis and Intragenesis
- cisgenesis **OR** cisgenic **OR** cisgene
- intragenesis **OR** intragenic **OR** intragene

47 The patent search was finalised in November 2010. Patent applications are published 18 months after filing. That means that only patents filed before February 2009 are included in the findings.

RNA-dependent DNA methylation (RdDM)
- transcriptional **AND** "gene silencing" **AND** TGS **AND** plant
- RdDM **AND** plant

Grafting (on GM rootstock)
- graft* **AND** rootstock* **AND** transgenic
- "transgenic rootstock*"
- "GM rootstock*"

Reverse Breeding
- "reverse breeding"

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)
- agroinfiltration **OR** "agro infiltration"
- agroinoculation **OR** "agro inoculation"
- agroinfection **OR** "agro infection"
- "vacuum infiltration" **AND** Agrobacterium

Patents on floral dip were not analysed further as plants derived from this technique do not differ from GM plants obtained by other transformation methods and therefore the technique is not considered as relevant for discussion.

Due to the long history of the use of agro-infiltration and floral dip and to diverse applications of the techniques in research, hundreds of patents were found by using the keywords above. In order to reduce the results to a more manageable number and to identify patents specifically focused on these techniques, the keyword search was performed in the claims only.

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.

Patents obtained were categorised according to:
- Priority date (date of first application);
- Country of applicant/s;
- Private or public applicant;
- Claimed plant/s;
- Claimed trait/s obtained through the application of the technique.

ANNEX 6: PATENT SEARCH – DETAILED RESULTS

The lists of patents identified for each new plant breeding technique are presented below together with tables reporting detailed data from the content analysis of patents. In particular, data on plants and traits claimed in patents are illustrated.

Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

Box 1 reports the results of the patent search for ZFN technology and Table 12 illustrates how ZFN patents are distributed in terms of plants and traits claimed and of type of technique employed (ZFN-3 for targeted insertion or ZFN-1 and -2 for targeted mutagenesis). Patents in which all three techniques are claimed or patents in which several types of plants or traits are claimed are counted more than once in the table. The same applies for the following tables.

Table 12: Plants and traits claimed in patents on ZFN technology.

PLANTS	TRAITS	targeted insertion (ZFN-3)	male sterility	targeted mutation (ZFN-1, -2)	herbicide tolerance	changed composition
plants in general		6	-	4	1	1
model plants		3	1	1	1	-
tobacco		2	1	1	1	-
Arabidopsis		2	-	-	-	-
crop plants		5	1	2	1	-
maize		2	-	1	-	-
ornamentals		1	1	1	1	-

BOX 1: PATENTS ON ZFN TECHNOLOGY

BIESGEN, C. (2001). Methods for the transformation of vegetal plastids, WO/03/054189. SunGene GmbH & Co. KGaA.

BUTLER, H., D. R. CORBIN, et al. (2009). Targeted integration into the Zp15 locus, WO/2010/077319. S. B. I. Dow AgroSciences LLC.

CAI, Q. C., J. MILLER, et al. (2006). Optimized non-canonical zinc finger proteins, WO/2008/076290 SANGAMO BIOSCIENCES INC & DOW AGROSCIENCES LLC.

CARROLL, D., M. BIBIKOVA, et al. (2002). TARGETED CHROMOSOMAL MUTAGENESIS USING ZINC FINGER NUCLEASES. UNIV UTAH RES FOUND [US].

DEKELVER, R., M. C. HOLMES, et al. (2008). LINEAR DONOR CONSTRUCTS FOR TARGETED INTEGRATION, WO/2009/131632. SANGAMO BIOSCIENCES INC [US].

GUPTA, M., A. PALTA, et al. (2007). ENGINEERED ZINC FINGER PROTEINS TARGETING 5-ENOLPYRUVYL SHIKIMATE-3-PHOSPHATE SYNTHASE GENES, WO/2009/042164. DOW AGROSCIENCES LLC [US] & SANGAMO BIOSCIENCES INC [US].

LILJEDAHL, M., S. E. ASPLAND, et al. (2002). METHODS AND COMPOSITIONS FOR USING ZINC FINGER ENDONUCLEASES TO ENHANCE HOMOLOGOUS RECOMBINATION, WO/03/080809.

LYZNIK, L. A., Y. TAO, et al. (2007). METHODS FOR ALTERING THE GENOME OF A MONOCOT PLANT CELL, WO/2009/006297. PIONEER HI BRED INT [US].

MILLER, J., W. M. AINLEY, et al. (2006). Zinc finger nuclease-mediated homologous recombination, WO/2008/021207 SANGAMO BIOSCIENCES INC & DOW AGROSCIENCES LLC.

MILLER, J. C. (2006). Engineered cleavage half-domains, US/2009/311787. Sangamo BioSciences Inc.

MILLER, J. C. (2008). Compositions for linking DNA-binding domains and cleavage domains, WO/2009/154686. Sangamo BioSciences Inc.

MILLER, J. C. and L. ZHANG (2004). METHODS AND COMPOSTIONS FOR TARGETED CLEAVAGE AND RECOMBINATION, WO/2005/084190. SANGAMO BIOSCIENCES INC [US].

PETOLINO, J., C. CAI, et al. (2008). PROTEIN PRODUCTION IN PLANT CELLS AND ASSOCIATED METHODS AND COMPOSITIONS, WO/2010/019386. S. B. I. U. DOW AGROSCIENCES LLC [US].

ROLLAND A., DUBALD M., et al. (2007). METHODS AND MEANS FOR EXACT REPLACEMENT OF TARGET DNA IN EUKARYOTIC ORGANISMS, WO/2008/148559, BAYER BIOSCIENCE NV [BE] & BAYER CROPSCIENCE SA [FR].

VAINSTEIN, A. and A. ZUKER (2008). PLANT VIRAL EXPRESSION VECTORS AND USE OF SAME FOR GENERATING GENOTYPIC VARIATIONS IN PLANT GENOMES, WO/2009/130695, DANZIGER INNOVATION LTD [IL].

WANG, J. (2008). METHODS AND COMPOSITIONS FOR TARGETED SINGLE-STRANDED CLEAVAGE AND TARGETED INTEGRATION, WO/2010/021692. SANGAMO BIOSCIENCES INC [US].

Oligonucleotide-directed mutagenesis (ODM)

Patents identified for ODM are listed in Box 2 and plants and traits claimed in ODM patents are shown in Table 13.

Table 13: Plants and traits claimed in patents on ODM.

PLANTS	TRAITS	targeted mutation in general	herbicide tolerance	**others:** disease resistance, dehiscence prevention, chromatine assembly
plants in general		13	2	3
tobacco		-	1	-
crop plants		-	7	-
maize		-	4	-
brassicaceae		1	3	-
ornamentals		-	2	-

BOX 2: PATENTS ON ODM

ANDREWS, W. H., M. J. MORSER, et al. (1991). NOVEL MUTAGENESIS METHODS AND COMPOSITIONS, WO/93/01282, BERLEX LAB [US].

ANDRUS, A. and R. G. KUIMELIS (1997). IMPROVED CHIMERIC OLIGONUCLEOTIDE VECTORS, WO/98/39353 PERKIN ELMER CORP [US].

ARNTZEN, C. J., P. B. KIPP, et al. (1997). USE OF MIXED DUPLEX OLIGONUCLEOTIDES TO EFFECT LOCALIZED GENETIC CHANGES IN PLANTS, WO/99/07865, KIMEAGEN INC [US].

BADUR, R. and B. REISS (2003). METHOD FOR PRODUCING RECOMBINANT ORGANISMS, WO/2004/085644, BASF PLANT SCIENCE GMBH [DE].

BASZCZYNSKI, C. L., J. H. DUESING, et al. (1997). TARGETED MANIPULATION OF HERBICIDE-RESISTANCE GENES IN PLANTS, WO/99/25853, PIONEER HI BRED INT [US].

BEETHAM, P., P. AVISSAR, et al. (1999). Compositions and methods for plant genetic modification, WO/01/25460, VALIGEN INC [US].

BRACHMAN, E., L. FERRARA, et al. (2004). METHODS AND KITS TO INCREASE THE EFFICIENCY OF OLIGONUCLEOTIDE-DIRECTED NUCLEIC ACID SEQUENCE ALTERATION, WO/2005/108622, UNIV DELAWARE [US].

BUNDOCK, P. (2007). TARGETED NUCLEOTIDE EXCHANGE WITH IMPROVED MODIFIED OLIGONUCLEOTIDES, WO/2009/002150, KEYGENE NV [NL].

BUNDOCK, P., M. DE BOTH, et al. (2005). IMPROVED TARGETED NUCLEOTIDE EXCHANGE WITH LNA MODIFIED OLIGONUCLEOTIDES, EP/2002/001, KEYGENE NV [NL].

BUNDOCK, P., M. DE BOTH, et al. (2007). AN IMPROVED MUTAGENESIS METHOD USING POLYETHYLENE GLYCOL MEDIATED INTRODUCTION OF MUTAGENIC NUCLEOBASES INTO PLANT PROTOPLASTS, WO/2009/082190, KEYGENE NV [NL].

GAMPER, H. B., E. KIMIEC, et al. (2000). BINARY HYBRID MUTATIONAL VECTORS, WO/01/94610, UNIV JEFFERSON [US] & UNIV MIAMI [US].

GOCAL, G., P. AVISSAR, et al. (2001). NON-TRANSGENIC HERBICIDE RESISTANT PLANTS, WO/03/013226, CIBUS GENETICS [US].

GOCAL, G. F. W., M. E. KNUTH, et al. (2006). EPSPS MUTANTS, WO/2007/084294, CIBUS LLC [US].

GOFF, S. A. (2001). Locked nucleic acid containing heteropolymers and related methods, US/2006/117410, SYNGENTA PARTICIPATIOUS AG [CH].

HAWKES, T. R., A. J. GREENLAND, et al. (1997). METHODS OF IN SITU MODIFICATION OF PLANT GENES, WO/98/54330, ZENECA LTD [GB].

KMIEC, E. B. (1996). CHIMERIC MUTATIONAL VECTORS HAVING NON-NATURAL NUCLEOTIDES, WO/97/48714, UNIV JEFFERSON [US] & UNIV MIAMI [US].

KMIEC, E. B., H. B. GAMPER, et al. (2000). Targeted chromosomal genomic alterations with modified single stranded oligonucleotides, EP/1268768, University of Delaware.

KMIEC, E. B., H. B. GAMPER, et al. (2000). Targeted chromosomal genomic alterations in plants using modified single stranded oligonucleotides, US/2003/236208, UNIV DELAWARE [US].

KMIEC, E. B., H. PAREKH-OLMEDO, et al. (2002). 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].

MAHAJAN, P. B. and P. KANNAN (2002). TARGETED MANIPULATION OF GENES IN PLANTS, WO/03/076574, PIONEER HI BRED INT [US].

MAY, G. D., E. B. KMIEC, et al. (2000). PLANT GENE TARGETING USING OLIGONUCLEOTIDES, WO/01/87914, UNIV DELAWARE [US].

PROKOPISHYN, N. L. (2002). Short fragment homologous recombination to effect targeted genetic alterations in plants, WO/03/062425, PROKOPISHYN NICOLE LESLEY [US].

RAINEY-WITTICH, D. Y., M. DE BOTH, et al. (2005). METHOD AND MEANS FOR TARGETED NUCLEOTIDE EXCHANGE, WO/2007/037676, KEYGENE NV [NL].

SCHOPKE, C., G. F. W. GOCAL, et al. (2007). MUTATED ACETOHYDROXYACID SYNTHASE GENES IN BRASSICA, WO/2009/046334, CIBUS LLC [US].

SUNDARESAN, V. and S. RAJANI (2000). DEHISCENCE GENE AND METHODS FOR REGULATING DEHISCENCE, WO/01/59122, INST OF MOLECULAR AGROBIOLOGY [SG].

Cisgenesis and Intragenesis

Box 3 reports results of the patent search for cisgenesis and intragenesis and Table 14 shows plants and traits claimed in the patents.

Table 14: Plants and traits claimed in patents on cisgenesis/intragenesis.

PLANTS / TRAITS	insertion of cis/intragene	changed composition	blackspot bruising tolerance	reduced cold-induced sweetening	pest resistance	fungi	nematodes
plants in general	4	-	-	-	-	-	-
tobacco	-	-	-	-	1	1	-
crop plants	2	3	1	1	9	8	1
wheat	-	-	1	1	-	-	-
solanaceae	-	3	1	1	9	8	1
potato	-	3	1	1	7	6	1
tomato	-	1	-	-	2	2	-

BOX 3: PATENTS ON CISGENESIS AND INTRAGENESIS

ALLEFS, J. J. H. M. and E. A. G. VAN DER VOSSEN (2002). GENE CONFERRING RESISTANCE TO PHYTOPHTHORA INFESTANS (LATE-BLIGHT) IN SOLANACEA, WO/03/066675, KWEEK EN RESEARCHBED AGRICO BV [NL].

CONNER, A., J. PRINGLE, et al. (2009). PLANT TRANSFORMATION USING DNA MINICIRCLES, WO/2010/090536, NEW ZEALAND INST FOR PLANT AND [NZ].

CONNER, A. J., P. J. BARRELL, et al. (2004). TRANSFORMATION VECTORS, WO/2005/121346, THE NEW ZEALAND INSTITUTE FOR PLANT AND FOOD RESEARCH LIMITED.

DE VETTEN, N. C. M. H., R. G. F. VISSER, et al. (2007). 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].

HALTERMAN, D. and Z. LIU (2007). LATE BLIGHT RESISTANCE GENE FROM WILD POTATO, WO/2009/023755 WISCONSIN ALUMNI RES FOUND [US].

JACOBSEN, E., R. G. F. VISSER, et al. (2007). Identification, classification and optionally stacking of r-genes in solanum using an effector-receptor approach, EP/1950304, COOEPERATIE AVEBE U A [NL].

JONES, J., S. J. FOSTER, et al. (2007). LATE BLIGHT RESISTANCE GENES AND METHODS, WO/2009/013468, WAGENINGEN UNIVERSITY [NL] & PLANT BIOSCIENCE LTD [GB].

LUO, J., E. BUTELLI, et al. (2008). METHODS AND COMPOSITIONS FOR MODIFYING PLANT FLAVONOID COMPOSITION AND DISEASE RESISTANCE, WO/2009/103960, NORFOLK PLANT SCIENCES LTD [GB].

OSUMI, T., W. R. BELKNAP, et al. (2002). SOLANUM BULBOCASTANUM LATE BLIGHT RESISTANCE GENE AND USE THEREOF, WO/2004/020594, US AGRICULTURE [US].

ROMMENS, C. (2004). PLANT-SPECIFIC GENETIC ELEMENTS AND TRANSFER CASSETTES FOR PLANT TRANSFORMATION, WO/2008/082429, SIMPLOT CO J R [US].

ROMMENS, C. (2005). Low acrylamide foods, WO/2007/035752, SIMPLOT CO J R [US].

ROMMENS, C., H. YAN, et al. (2007). REDUCED ACRYLAMIDE PLANTS AND FOODS, US/2009/123626, SIMPLOT CO J R [US].

ROMMENS, C. M. T., J. YE, et al. (2002). PRECISE BREEDING, WO/03/069980, SIMPLOT CO J R [US].

VAN DER VOSSEN, E. A. G., A. A. LOKOSSOU, et al. (2007). A FUNCTIONAL R-GENE FROM SOLANUM BULBOCASTANUM, WO/2008/091153, WAGENINGEN UNIVERSITEIT [NL] & KWEEK EN RESEARCHBED AGRICO BV (NL).

VAN DER VOSSEN, E. A. G., J. N. VAN DER VOORT, et al. (1998). ENGINEERING NEMATODE RESISTANCE IN SOLANACAE, WO/0006754, WAGENINGEN UNIVERSITY [NL].

WEEKS, T. J. and C. M. T. ROMMENS (2003). REFINED PLANT TRANSFORMATION, WO/03/079765, SIMPLOT CO J R [US].

RNA-dependent DNA methylation (RdDM)

One patent on RdDM has been identified after a thorough search (Box 4). No specific plant species are claimed. 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.

BOX 4: PATENTS ON RDDM

WASSENEGGER, M., G. KRCZAL, et al. (2008). METHOD FOR THE PRODUCTION OF A TRANSGENE FREE PLANT WITH ALTERED METHYLATION PATTERN, WO/2010/066343, RLP AGROSCIENCE GMBH [DE].

Grafting (on GM rootstock)

Box 5 lists the patents identified on grafting on GM rootstock and Table 15 summarises the claims of the patents in terms of plants and traits.

Table 15: Plants and traits claimed in patents about grafting on GM rootstock.

PLANTS / TRAITS	gene silencing	change plant architecture	pest resistance	fungi	virus	bacteria	insects	nematodes
plants in general	1	1	-	-	-	-	-	-
crop plants	-	-	11	1	8	1	1	1
cucumber	-	-	1	-	1	-	-	-
grapevine	-	-	5	-	4	-	1	-
apple	-	-	2	-	-	1	1	-
pear	-	-	1	-	-	1	-	-
tomato	-	-	1	-	1	-	-	-
citrus	-	-	3	-	3	-	-	-
beet	-	-	1	-	1	-	-	-
tobacco	-	-	1	-	1	-	-	-
maize	-	-	1	-	-	-	1	-
soybean	-	-	1	-	-	-	-	1
conifer	-	-	1	-	1	-	-	-

BOX 5: PATENTS ON GRAFTING ON GM ROOTSTOCK

ALDWINCKLE, H. S. and J. L. NORELLI (1992). TRANSGENIC POMACEOUS FRUIT WITH FIRE BLIGHT RESISTANCE, WO/94/07356 CORNELL RES FOUNDATION INC [US].

ALLEN, E., W. P. DONOVAN, et al. (2007). INVERTEBRATE MICRORNAS, WO/2008/103643, MONSANTO TECHNOLOGY LLC [US].

CZOSNEK, H. (2007). VIRUS TOLERANT PLANTS AND METHODS OF PRODUCING SAME, WO/2008/102337, YISSUM RES DEV CO [IL].

GAL-ON, A., A. ZELCER, et al. (2004). ENGRAFTED PLANTS RESISTANT TO VIRAL DISEASES AND METHODS OF PRODUCING SAME, WO/2005/079162 ISRAEL STATE [IL].

GMITTER, F. G., Z. DENG, et al. (2001). CITRUS TRISTEZA VIRUS RESISTANCE GENES AND METHODS OF USE, WO/03/068911, UNIV FLORIDA [US].

GONSALVES, D. and K. LING (1995). GRAPEVINE LEAFROLL VIRUS PROTEINS AND THEIR USES, WO/97/22700 CORNELL RES FOUNDATION INC [US].

GONSALVES, D. and B. MENG (1997). RUPESTRIS STEM PITTING ASSOCIATED VIRUS NUCLEIC ACIDS, PROTEINS, AND THEIR USES, WO/98/52964, CORNELL RES FOUNDATION INC [US].

GONSALVES, D., B. XUE, et al. (1997). NEPOVIRUS RESISTANCE IN GRAPEVINE, WO/99/16298, CORNELL RES FOUNDATION INC [US].

IVASHUTA, S. I., B. E. WIGGINS, et al. (2008). RECOMBINANT DNA CONSTRUCTS AND METHODS FOR MODULATING EXPRESSION OF A TARGET GENE, WO/2010/002984, MONSANTO TECHNOLOGY LLC [US].

POLSTON, J. E. and E. HIEBERT (2004). MATERIALS AND METHODS FOR PROVIDING RESISTANCE TO PLANT PATHOGENS IN NON-TRANSGENIC PLANT TISSUE, WO/2005/118805, UNIV FLORIDA [US].

SCHMULLING, T. and T. WERNER (2001). METHOD FOR MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY, WO/03/050287.

SCHNABEL, G., R. Scorza, et al. (2006). INCREASED RESISTANCE OF PLANTS TO PATHOGENS FROM MULTIPLE HIGHER-ORDER PHYLOGENETIC LINEAGES, CLEMSON UNIVERSITY RESEARCH FOUNDATION.

ZHU, H., K. LING, et al. (1997). GRAPEVINE LEAFROLL VIRUS (TYPE 2) PROTEINS AND THEIR USES, WO/98/53055, CORNELL RES FOUNDATION INC [US].

Reverse Breeding

Two patents were identified on reverse breeding (Box 6). In both cases, the invention is claimed for plants in general, without mentioning 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.

BOX 6: PATENTS ON REVERSE BREEDING

DIRKS, R. H. G., C. M. P. VAN DUN, et al. (2001). REVERSE BREEDING, WO/03/017753, RIJK ZWAAN ZAADTEELT EN ZAADHA [NL].

VAN DUN, C. M. P. and R. H. G. DIRKS (2005). NEAR REVERSE BREEDING, WO/2006/094773, RIJK ZWAAN ZAADTEELT EN ZAADHA [NL].

Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation)

Eleven patents were identified in which agro-infiltration is used for the high level expression of useful recombinant proteins (Box 7). Table 16 illustrates which plants and which recombinant proteins are claimed in those patents.

Patents on floral dip have not been analysed further as plants derived from this technique do not differ from GM plants obtained by other transformation methods and therefore the technique is not considered as relevant for discussion.

Table 16: Plants and traits claimed in patents on agro-infiltration.

PLANTS	TRAITS	production of recombinant proteins in general	antibodies	vaccines	pharmaceuticals	enzymes
plants in general		-	1	-	2	-
dicots		-	-	-	1	-
tobacco		3	1	1	1	2

BOX 7: PATENTS ON AGRO-INFILTRATION

BAULCOMBE, D. C., O. VOINNET, et al. (1999). ENHANCED EXPRESSION, WO/01/38512, PLANT BIOSCIENCE LTD [GB].

BENDAHMANE, A., B. STURBOIS, et al. (2004). METHOD FOR PRODUCING HIGHLY SENSITIVE ENDONUCLEASES, NOVEL PREPARATIONS OF ENDONUCLEASES AND USES THEREOF, WO/2006/010646, AGRONOMIQUE INST NAT RECH [FR] (INRA) & GENOPLANTE VALOR S A S [FR].

DOROKHOV, Y. L. and T. V. KOMAROVA (2007). METHOD FOR OVERPRODUCING ANTI-HER2/NEU ONCOGENE ANTIBODIES IN PLANT, WO/2009/048354, INST FIZ KHIM BIOLOG IM A N BE [RU] & FEDERAL NOE GUP G NTS NII ORCH [RU].

GALBA, P., C. M. POZZI, et al. (2008). PRODUCTION OF NGF IN PLANT, WO/2010/038158, FOND PARCO TECNOLOGICO PADANO [IT].

LINDBO, J. A. (2007). SYSTEM FOR EXPRESSION OF GENES IN PLANTS FROM A VIRUS-BASED EXPRESSION VECTOR, WO/2008/094512, OHIO SATE UNIVERSITY RES FOUND [US].

MARILLONNET, S., C. ENGLER, et al. (2004). BIOLOGICALLY SAFE TRANSIENT PROTEIN EXPRESSION IN PLANTS, WO/2006/003018, ICON GENETICS AG [DE].

MCDONALD, K. A., A. DANDEKAR, et al. (2006). CHEMICALLY INDUCIBLE CUCUMBER MOSAIC VIRUS PROTEIN EXPRESSION SYSTEM, WO/2008/036424, UNIV CALIFORNIA [US].

MCDONALD, K. A., B. E. LINDENMUTH, et al. (2008). PRODUCTION OF CELLULASE ENZYMES IN PLANT HOSTS USING TRANSIENT AGROINFILTRATION, WO/2010/022186, UNIV CALIFORNIA [US].

NEGROUK, V., G. NEGROUK, et al. (2002). TRANSIENT PRODUCTION OF PHARMACEUTICALLY IMPORTANT PROTEINS IN PLANTS, WO/2005/076766, SUNOL MOLECULAR CORP [US] & ALTOR BIOSCIENCE CORP [US].

WEISSINGER, A., K. AZHAKANANDAM, et al. (2005). METHODS AND COMPOSITIONS FOR EXPRESSING PROTEINS IN PLANTS, WO/2007/005882, UNIV NORTH CAROLINA STATE [US].

WILLIAMSON, A., E. P. RYBICKI, et al. (2005). EXPRESSION OF PROTEINS IN PLANTS, WO/2006/119516, UNIV CAPE TOWN [ZA].

ANNEX 7: FIELD TRIALS - METHODOLOGY

We have evaluated the applications for field trials submitted in the EU under Directive 2001/18/EC between October 2002 and July 2010. 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 the summary of the notifications 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.

In our search, we relied 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.

It was possible to identify field trials for products of cisgenesis/intragenesis and grafting on GM rootstock. We did not identify notifications for crops obtained by other new plant breeding techniques. However, as the commercialised crops produced by these techniques in most of the cases do not posses stably inserted genes, it might not be possible to identify respective field trials correctly, because of lack of detailed information on the applied method.

ANNEX 8: FIELD TRIALS – DETAILED RESULTS

On the basis of information on the genetic modification provided in the notification applications for the following new plant breeding techniques could be identified:

TECHNIQUE	INSTITUTE/ COMPANY	MEMBER STATE	NOTIFICATION NUMBER	YEARS	PLANT	TRAIT	GENE INSERTED
INTRAGENESIS	AVEBE	NL	B/NL/07/05	2008-2011	potato	reduced amylose content	Granule-bound starch synthase (GBSS)
			B/NL/04/04	2004-2013			
			B/NL/03/04	2004-2013			
			B/NL/07/04	2008-2015			
CISGENESIS[48]	Wageningen University	NL	B/NL/07/01	2007-2012	potato	late blight resistance	Rpi-blb1 gene for late blight resistance
			B/NL/09/02	2010-2020			
GRAFTING ON GM ROOTST.	INRA	FR	B/FR/09/11/01	2010-2014	grape	resistance to Grapevine fanleaf virus (GFLV)	coat protein (CP) gene of GFLV
			B/FR/04/05/01	2004-2008	grape (vinifera x berlandieri)		
GRAFTING ON GM ROOTST.	IVIA	ES	B/ES/06/43	2007-2017	carrizo citrange	modified plant architecture	GA20-oxidase gene
			B/ES/08/03	2008-2018			
GRAFTING ON GM ROOTST.	IVIA	ES	B/ES/08/04	2008-2018	sweet orange	tolerance to Phytophtora citrophtora	PR P23 gene for tolerance to Phytophtora
GRAFTING ON GM ROOTST.	Swedish University of Agr. Sciences	SE	B/SE/04/1227	2004	apple pear	improved rooting ability	rolB gene
			B/SE/09/12183	2010-2014	apple pear		
GRAFTING OF GM SCION	PRI	NL	B/NL/02/03	2003-2007	apple	resistance to fungi	hth: alpha-hordothionine gene
			B/NL/04/02	2004-2008			

48 According to the information provided in the notification the genome contains T-DNA borders from *Agrobacterium*.

ANNEX 9: DEFINITIONS OF PLANT BREEDING TECHNIQUES

Agro-infiltration:

Plant tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium sp.* containing a genetic construct. The genetic construct is locally expressed at high level, without being integrated into the plant genome.

Cell fusion/Protoplast fusion[49]:

Protoplasts are produced by removing the cell wall from plant cells using either mechanical or enzymatic means. Protoplasts from two different species can be fused to create a hybrid. The fusion can be accomplished by an electrical process or by chemical agents.

Cisgenesis and intragenesis:

A DNA fragment from the species itself or from a cross-compatible species is inserted into the plant genome. In the case of cisgenesis, the inserted gene is unchanged with its own introns and regulatory sequences. 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.

Dihaploid breeding:

Dihaploids are used for breeding crops that are natural polyploids (e.g. potato with four basic sets of chromosomes, 4n). A dihaploid plant (in this case 2n) is generated and is used for any type of breeding (conventional or biotechnology) since breeding and crossings with polyploids are extremely complex. At the end of the breeding process the polyploidy is restored.

Double haploid breeding:

A haploid plant is generated out of pollen grains with one set of chromosomes (n) followed by duplicating the chromosomes to generate a 2n plant. This is a way to obtain 100% homozygous individuals which can be used as parental lines for hybrid production.

Embryo rescue:

In the case of wide crosses, the embryo formed after fertilisation frequently fails to develop. When applying the technique of embryo rescue, the ovary is excised within several days after fertilisation to avoid abortion. The embryo is then nurtured into a full plant by using the tissue culture technology.

Genomic-assisted breeding:

Genomic-assisted breeding developed from marker-assisted breeding. It aims at rapidly investigating the genetic makeup of individual plants and selecting desirable genotypes by using diverse molecular-based tools.

Grafting (on GM rootstock):

A chimeric plant is produced by grafting a non-genetically modified scion on a genetically modified rootstock.

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49 Protoplast fusion of two or more cells by means of methods that do not occur naturally is a technique of genetic modification (Directive 2001/18/EC, Annex 1A, Part 1 (3)). Protoplast fusion of plant cells of organisms which can exchange genetic material through traditional breeding methods is a technique of genetic modification yielding organisms to be excluded from the Directive (Directive 2001/18/EC, Annex 1B (2)).

In vitro fertilization[50]:

Plant reproductive structures such as flower explants, ovaries, ovules and mature pollen, are isolated. Fusion of gametes is achieved in suitable solutions *in vitro* and can be facilitated by the presence of chemicals such as calcium ions or polyethylene glycol (PEG) or an by electroporation. This allows the production of hybrids even between only remotely related species.

Meganuclease delivered as DNA; meganuclease delivered as RNA; meganuclease delivered as protein:

Meganucleases are proteins that specifically recognize target DNA sequences of 12 to over 30 base pairs and create a double strand break (DSB) that activates repair mechanisms and DNA recombination. Similarly to ZFNs, the technique can be used for site-specific mutagenesis or for targeted gene insertion by homologous recombination. Newly designed meganucleases can be produced in order to induce site-specific DNA recombination at a chosen locus in plant cell.

Mutagenesis[51]:

Chemicals such as ethyl methane sulfonate (EMS) or ionising radiations are used to cause random mutation in the DNA of crops. The treated plants are screened for interesting properties.

Oligonucleotide directed mutagenesis (ODM):

Also known as Targeted Gene Repair, Oligonucleotide-directed Gene Targeting, Genoplasty, Chimeraplasty, etc.

Oligonucleotides target homologous DNA and induce site-specific nucleotide substitutions, insertions or deletions through repair mechanisms. The following types of oligonucleotides are used: Single stranded DNA oligonucleotides, chimeric oligonucleotides, triple helix-forming oligonucleotides (TFOs) and RNA oligonucleotides.

Polyploidy induction[52]:

Polyploidy occurs in cells when there are more than two paired sets of chromosomes. It can be induced in cell culture by some chemicals e.g. colchicine.

Reverse breeding:

Homozygous parental lines are produced from selected heterozygous plants by suppressing meiotic recombination. This suppression is obtained through RNA interference-mediate downregulation of genes involved in the meiotic recombination process. Subsequently, the obtained homozygous lines are hybridised, in order to reconstitute the original genetic composition of the selected heterozygous plants.

RNA-dependent DNA methylation (RdDM):

Genes encoding RNAs which are homologous to plant sequences, like promoter regions, are delivered to the plant cells. These genes, once transcribed, give rise to the formation of small double stranded RNAs. They induce methylation of the homologous sequences and consequently inhibit their transcription.

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50 Not considered to result in genetic modification (Directive 2001/18/EC, Annex 1A, part 2 (1)).
51 Technique of genetic modification yielding organisms to be excluded from the Directive (Directive 2001/18/EC, Annex 1B (1)).
52 Not considered to result in genetic modification (Directive 2001/18/EC, Annex 1A, Part 2 (3)).

Transgenesis[53]:

A DNA fragment from a non-cross compatible species is inserted into the plant genome.

Transgenic inducer construct-driven breeding tools:

A transgene encoding an RNAi construct or a dominant-negative protein is present in (e.g. inserted into the genome of) an inducer line. The expression of the transgene leads to the inhibition of gene expression or the inhibition of a protein function, respectively, thereby interfering with processes underlying to relevant biology. Interference with plant biology leads to the induction of the formation of materials enhancing breeding (e.g. biodiversity, recombination, haploids). The inducer transgene is segregated out during further breeding and therefore not present in the final product.

Zinc finger nuclease technology 1:

Genes encoding Zinc Finger Nucleases (ZFN) are delivered to plant cells without a repair template. The ZFN binds to the DNA and generates a site-specific double strand break (DSB). The natural DNA-repair process through non-homologous end-joining (NHEJ) leads to site-specific random mutations, which consist of changes of single or few base pairs, short deletions or insertions.

Zinc finger nuclease technology 2:

Genes encoding Zinc Finger Nucleases (ZFN) are delivered to plant cells along with a short repair template. The ZFN binds to the DNA and generates a site-specific double strand break (DSB). Gene repair mechanisms generate site-specific point mutations like changes of single or few base pairs through homologous recombination.

Zinc finger nuclease technology 3:

Genes encoding Zinc Finger Nucleases (ZFN) are delivered to plant cells along with a large stretch of DNA, whose ends are homologous to the DNA sequences flanking the cleavage site. As a result, the DNA stretch is site-specifically inserted into the plant genome.

53 Transgenesis (Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation) is a technique of genetic modification (Directive 2001/18/EC, Annex 1A, part 1 (1)).

ANNEX 10: WORKSHOP - PARTICIPANTS

LIST OF PARTICIPANTS

European Commission DGs and EU Authorities

- JRC Institute for Prospective Technological Studies: Jacques Delincé, Emilio Rodríguez Cerezo, Maria Lusser, Claudia Parisi, Marta Czarnak-Klos, Stephen Langrell
- DG Health and Consumers: Paula Rey Garcia
- JRC Unit Work Programme and Strategy: Anne-Katrin Bock
- JRC Institute for Health and Consumer Protection: Marc van den Bulcke
- JRC Institute for Reference Materials and Measurements: Philippe Corbisier
- DG Research: Jens Hoegel
- European Food Safety Authority (EFSA) Nancy Podevin

International organisations

- OECD David B. Sawaya

National regulators and public administration

- DEFRA, UK: Louise Ball
- Federal Office of Consumer Protection and Food Safety, Germany: Hans-Jörg Buhk
- ILVO-T&V, Belgium: Marc de Loose
- National Institute for Public Health and the Environment, The Netherlands: Boet Glandorf
- Scientific Institute of Public Health, Belgium: Philippe Herman

Public research

- Institut national de recherche agronomique: Pere Mestre
- Leiden University: Paul Hooykaas
- VU-University Amsterdam: Jan Kooter
- Wageningen University and Research Centre: Henk Schouten

Stakeholders associations

- Copa – Cogeca: Arnaud Petit
- EuropaBio: Filip Cnudde
- German Plant Breeders' Association: Petra Jorasch
- Union Française des Semenciers: Olivier Lucas

Private companies

- BASF PLANT SCIENCE HOLDING GmbH: Matthias Pohl
- Bayer BioScience N.V.: Stefania Meloni
- Bayer BioScience N.V.: Adrian Peres
- Cellectis S.A.: Mathis Luc
- Dow AgroSciences: Gaston Legris
- DU PONT PIONEER Overseas Corporation: Wim Broothaerts
- Eurosemillas S.A.: José Pellicer España
- GROUPE LIMAGRAIN HOLDING: Alain Toppan
- HZPC Holland B.V.: Robert Graveland
- Keygene N.V.: Arjen J. Van Tunen
- Monsanto: Jim Masucci
- Patent Attorney: Tim Roberts
- Rijk Zwaan Breeding B.V.: Kees Reinink
- Syngenta: Esteban Alcalde
- Zeta Seeds: Jesus Abad

ANNEX 11: WORKSHOP - AGENDA

Workshop on New plant breeding techniques: Adoption and economic impact

27 & 28 May 2010

European Commission (EC), Joint Research Centre (JRC)
Institute for Prospective Technological Studies (IPTS)
Venue: Edificio Expo, Room 116, calle Inca Garcilaso 3, 41092 Seville, Spain

Organisers: Maria Lusser (maria.lusser@ec.europa.eu)
Emilio Rodríguez Cerezo (emilio.rodriguez-cerezo@ec.europa.eu)

AGENDA

Thursday 27 May 2010 · Morning (9:00–13:30)		
Time	**Programme items**	**Speaker**
9:00-9:10	Welcome	Jacques Delincé, IPTS
9:10-9:15	Introduction to the workshop	Emilio Rodríguez Cerezo, IPTS
9:15-9:30	New plant breeding techniques - DG SANCO's approach	Paula Rey Garcia EC, Directorate-General Health and Consumers
9:30-9:45	Introduction to the project "New plant breeding techniques: Adoption and economic impact"	Maria Lusser, IPTS
	Horizontal presentations on new plant breeding techniques	
9:45-10:00	Practical application of advanced breeding technologies for crop improvement	Esteban Alcalde Syngenta, ES
10:00-10:15	Why innovation in plant breeding is needed: The importance of biotech and non-biotech breeding methods	Petra Jorasch German Plant Breeders' Association
10:15-10:30	New breeding techniques and transgenesis for an innovative agriculture	Olivier Lucas, UFS French Seed Association
10:30-10:45	Agricultural biotechnologies to 2030	David Sawaya, OECD, FR
10:45-11:00	Discussion	
11:00-11:30	*Coffee break*	
11:30-11:45	Proprietary rights for the products of new breeding techniques	Tim Roberts Patent Attorney, UK
11:45-12:00	New plant breeding techniques - innovation in the context of the EU legislative framework	Filip Cnudde EuropaBio, BE
12:00-12:15	Produce more and better: a need for the EU farming sector	Arnaud Petit Copa–Cogeca, BE
12:15-12:30	Biotechnology as a critical tool for vegetable breeding in the framework of the industry-university collaboration in Spain	Jesús Abad Martín Zeta Seeds, ES
12:30-12:45	Public-private platforms - a tool to strengthen the use of new technologies	José Pellicer España Eurosemilla, ES
12:45-13:05	Discussion	

	Synthetic Biology	
13:05-13:20	SynBio versus genetic engineering, are there new biosafety issues?	Hans-Jörg Buhk Federal Office of Consumer Protection and Food Safety, DE
13:20-13:30	Discussion	
13:30-14:30	Lunch break	
Thursday 27 May 2010 · Afternoon (14:30-18:30)		
Time	**Programme items**	**Speaker**
	Zinc Finger Nuclease Technique	
14:30-14:40	Zinc Finger Nuclease Technique 1-3: Definition/description	Boet Glandorf, National Institute for Publich Health and the Environment, NL
14:40-14:55	Efficient gene targeting by ZFNs	Paul Hooykaas Leiden University, NL
14:55-15:10	Delivering targeted mutagenesis: The use of zinc finger nucleases in plant breeding	Gaston Legris Dow AgroSciences, UK
15:10-15:25	Discussion	
	RNA dependent DNA methylation via RNA/siRNA	
15:25-15:30	RNA dependent DNA methylation via RNA/siRNA: Definition/description	Boet Glandorf, National Institute for Publich Health and the Environment, NL
15:30-15:45	Epigenetic modification of the plant genome: background, applications and consequences	Jan Kooter, VU-University Amsterdam, NL
15:45-16:00	RNA dependent DNA methylation via RNAi/siRNA	Jim Masucci Monsanto, USA
16:00-16:15	Discussion	
16:15-16:45	*Coffee break*	
	Reverse breeding	
16:45-16:50	Reverse breeding: Definition/description	Boet Glandorf, National Institute for Publich Health and the Environment, NL
16:50-17:05	Reverse breeding: an innovation tool for plant breeders	Stefania Meloni Bayer, BE
17:05-17:20	Reverse breeding applications in plant breeding and genetic research	Kees Reinink Rijk Zwaan, NL
17:20-17:30	Discussion	
	Agroinfiltration	
17:30-17:35	Agroinfiltration: Definition/description	Louise Ball DEFRA, UK
17:35-17:50	Agroinfiltration as a tool for the analysis of gene function in plants	Pere Mestre INRA, FR
	Grafting	
17:50-17:55	Grafting: Definition/description	Louise Ball DEFRA, UK
17:55-18:10	Plant grafting in the new biotechnology era	Adrian Peres Bayer, BE
18:10-18:30	Discussion	

Friday 28 May 2010 · 9:00–15:10		
Time	**Programme items**	**Speaker**
	Cisgenesis	
9:00-9:05	Cisgenesis: Definition/description	Louise Ball DEFRA, UK
9:05-9:20	Food and feed safety aspects of cisgenic crop plant varieties	Esther van Leeuwe-Kok RIKILT, NL
9:20-9:35	Cisgenesis for crop improvement	Henk Schouten Wageningen University, NL
9:35-9:50	Cisgenesis: possible exemptions?	Alain Toppan Limagrain, FR
9:50-10:05	Discussion	
	Oligonucleotide Gene Mutation	
10:05-10:20	Oligo-mediated mutagenesis: Basic principles, regulatory and safety issues	Philippe Herman Scientific Institute of Public Health, BE
10:20-10:35	Oligo Directed Mutagenesis: an efficient and natural mutagenesis method	Arjen van Tunen Keygene, NL
10:35-10:50	Targeted Mutagenesis as a tool to develop plant traits	Matthias Pohl BASF, DE
10:50-11:05	Discussion	
11:05-11:30	*Coffee break*	
	Further plant breeding techniques	
11:30-11:45	Meganucleases for the precise engineering of plant genomes.	Luc Mathis Cellectis, FR
11:45-12:00	Hybrid Technology	Wim Broothaerts Pioneer Hi-Bred Intl, BE
12:00-12:15	New traits through tilling	Robert Graveland HZPC Holland BV, NL
12:15-12:30	Discussion	
12:30-13:30	*Lunch break*	
	Preliminary results and further steps in the project	
13:30-13:50	New plant breeding techniques: Results of literature search	Claudia Parisi, IPTS
13:50-14:05	New techniques and changes in the genome	Marc de Loose ILVO-T&V, BE
14:05-14:20	New techniques and detection challenges	Marc de Loose ILVO-T&V, BE
	Further developments	
14:20-14:35	New plant breeding techniques from the DG RTD perspective	Jens Hoegel, EC Directorate-General Research
14:35-15:10	Final discussion	

ANNEX 12: SURVEY - METHODOLOGY

A survey was carried out through a questionnaire[54]. The draft questionnaire was sent to colleagues of the Commission Services and the private sector for comments and revised accordingly.

The survey was directed to companies using biotechnology for plant breeding and biotechnology companies providing techniques for plant breeders. Suitable companies were identified with the support of European and national seed breeders associations and on the basis of information from the Internet. The companies were contacted directly or through seed breeders associations to clarify if they used biotechnology and if they were prepared to participate in the survey. Only one branch each from international groups was included in the survey to avoid duplication of answers.

The questionnaire was sent to 27 companies and was returned completed by 18 companies (67%). One of the questionnaires was excluded from the evaluation as answers were received from two branches of the same international group. The evaluation of the answers is reported in section 5.4. The results are presented in an aggregate form to guarantee the confidentiality of the received information.

The answers of questions concerning the main constraints and benefits were evaluated after compiling them for all techniques. When the evaluation of the answers is carried separately for each of the techniques, they do not show clear tendencies because of the low sample number.

54 See Annex 13.

ANNEX 13: SURVEY – QUESTIONNAIRE

Questionnaire: new techniques for plant breeding

We would appreciate your response by 30 April 2010, preferably by returning this completed form by e-mail (maria.lusser@ec.europa.eu), fax (+34.95.448.84.34) or post[55].

Your response will be treated as confidential. The information will only be used within this study and aggregated for analysis. The European Commission is committed to data protection and privacy[56].

It will take about 20-40 minutes to complete the questionnaire (depending on the number of new plant breeding techniques used by your company).

We will report on the survey as a part of the JRC project "New plant breeding techniques: Adoption and economic impact". We will send the draft final report for comments to all participants in the survey (please make sure that you have provided your e-mail address below).

Thank you very much for your contribution!

Name of the company you are responding for: _____

Home country: _____

Its primary sectors of activity: _____

Your name: _____

Job title: _____

E-mail: _____

Phone number: _____

The European Commission plans to clarify trends revealed in the analysis, which may involve short follow-up interviews. Please **tick here** □ if you do not wish to be approached for this purpose.

A. CORPORATE BACKGROUND:

1. The company is

The branch of an international group □

An independent company □

Other □ please specify:_____

55 European Commission, Institute for prospective Technological Studies (IPTS), Attn.: Maria Lusser, Edificio EXPO, Calle Inca Garcilaso s/n, E-41092, Spain, Tel.: +34.95.404.85.51

56 See Disclaimer on page 6.

2. If the company is the branch of an international group: In which country is the mother company situated?

In _____.

3. What was the turnover of the company in the last financial year?

About euro _____million for the financial year ending _____.

4. If the company is the branch of an international group: What was the turnover of the whole group in the last financial year?

About euro _____million for the financial year ending _____.

5. How many employees work in the company?

About _____.

6. If the company is the branch of an international group: How many employees work in the whole group?

About _____.

B. FIELD OF BUSINESS:

7. The focus of the company is

Technology provider for plant breeders □

Plant breeding □

Other □ specify:_____

8. If the company focuses on plant breeding: What are the main crops?

Please specify the commodities:

_____; about _____%

_____; about _____%

_____; about _____%

_____; about _____%

_____; about _____%

C. USE OF BIOTECHNOLOGY FOR PLANT BREEDING

9. Are the following "established" plant breeding techniques used by the company?

	yes	no
Transgenesis (a)	□	□
Marker assisted selection (b)	□	□
Others (please specify)		
_____	□	□
_____	□	□
_____	□	□
_____	□	□

(a) Transgenesis:
A DNA fragment from a non-cross compatible species is inserted into the plant genome.

(b) Marker assisted selection:
After hybridisation, plants with traits of interest are selected by identifying marker genes linked to those traits.

10. Are the following "new" plant breeding techniques used by the company?

	yes	no
Zinc finger nuclease technology 1 (a)	□	□
Zinc finger nuclease technology 2 (b)	□	□
Zinc finger nuclease technology 3 (c)	□	□
Oligonucleotide-directed mutagenesis (d)	□	□
Cisgenesis/Intragenesis (e)	□	□
RNA dependent DNA methylation via RNAi/siRNA (f)	□	□
Grafting on a genetically modified rootstock (g)	□	□
Reverse breeding (h)	□	□
Agro-infiltration (i)	□	□
Other (please specify):		
_____	□	□
_____	□	□
_____	□	□
_____	□	□

(a) **Zinc finger nuclease technology 1:** Genes encoding for Zinc Finger Nucleases (ZFN) are delivered to plant cells without a repair template. The ZFN binds to the DNA and generates a site-specific double strand break (DSB). The natural DNA-repair process through non-homologous end-joining (NHEJ) leads to site-specific random mutations, which consist of changes of single or few base pairs, short deletions or insertions.

(b) **Zinc finger nuclease technology 2:** Genes encoding for Zinc Finger Nucleases (ZFN) are delivered to plant cells along with a short repair template. The ZFN binds to the DNA and generates a site-specific double strand break (DSB). Gene repair mechanisms generate site-specific point mutations like changes of single or few base pairs through homologous recombination.

(c) **Zinc finger nuclease technology 3:** Genes encoding for Zinc Finger Nucleases (ZFN) are delivered to plant cells along with a large stretch of DNA, whose ends are homologous to the DNA sequences flanking the cleavage site. As a result, the DNA stretch is site-specifically inserted into the plant genome.

(d) **Oligonucleotide-directed mutagenesis:** Also known as Targeted Gene Repair, Oligonucleotide-directed Gene Targeting, Genoplasty, Chimeraplasty, etc. Oligonucleotides target homologous DNA and induce site-specific nucleotide substitutions, insertions or deletions through repair mechanisms. The following types of oligonucleotides are used: Single stranded DNA oligonucleotides, chimeric oligonucleotides, triple helix-forming oligonucleotides (TFOs) and RNA oligonucleotides.

(e) **Cisgenesis/Intragenesis:** A DNA fragment from the species itself or from a cross compatible species is inserted into the plant genome. In the case of cisgenesis, the inserted gene is unchanged and flanked by its own introns and regulatory sequences. 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.

(f) **RNA dependent DNA methylation via RNAi/siRNA:** Genes encoding for RNAs which are homologous to plant sequences, like promoter regions, are delivered to the plant cells. These genes, once transcribed, give rise to the formation of small double stranded RNAs. They induce methylation of the homologous sequences and consequently inhibit their transcription.

(g) **Grafting on a genetically modified rootstock:** A chimeric plant is produced by grafting a non-genetically modified scion on a genetically modified rootstock.

(h) **Reverse breeding:** Homozygous parental lines are produced from selected heterozygous plants by suppressing meiotic recombination. This suppression is obtained through RNA interference-mediate downregulation of genes involved in the meiotic recombination process. Subsequently, the obtained homozygous lines are hybridised, in order to reconstitute the original genetic composition of the selected heterozygous plants.

(i) **Agro-infiltration:** Plant tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium sp.* containing a genetic construct. The genetic construct is locally expressed at high level, without being integrated into the plant genome.

11. If you answered "yes" in tables 9 and/or 10, please provide detailed information on the phase of implementation if available:

	CROP(S)/TRAIT(S)	RESEARCH	DEVELOPMENT				COMMERCIA-LISATION (e)
			PHASE I (a)	PHASE II (b)	PHASE III (c)	PHASE IV (d)	
Transgenesis	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Marker assisted selection	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Zinc finger nuclease technology 1	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Zinc finger nuclease technology 2	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Zinc finger nuclease technology 3	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Oligonucleotide-directed mutagenesis	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Cisgenesis/Intragenesis	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
RNA dependent DNA methylation via RNAi/siRNA	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐

Grafting on a genetically modified rootstock	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Reverse breeding	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Agro-infiltration	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
Others (please specify):	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
_____	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
_____	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
_____	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐
	_____	☐	☐	☐	☐	☐	☐

(a) PHASE I: Gene optimisation, crop transformation
(b) PHASE II: Trait development, pre-regulatory data, large-scale transformation
(c) PHASE III: Trait integration, field testing, regulatory data generation (if applicable)
(d) PHASE IV: Regulatory submission (if applicable), seed bulk-up, pre-marketing
(e) commercialisation: in the EU (where appropriate) or in non EU countries

12. If you answered “yes” to the use of “new” plant breeding techniques in table 10, in which areas do you see the major technical and socio economic constraints for development and commercialisation of the new plant breeding techniques? Please assess the relevance of each factor of the following list.

12 (a) Please specify technique: _____

Main constraints	Very high	High	Average	Low	Do not know
Cost of technology	☐	☐	☐	☐	☐
Intellectual property rights	☐	☐	☐	☐	☐
Legal situation	☐	☐	☐	☐	☐
Acceptance of consumers/users	☐	☐	☐	☐	☐
Other (please specify):	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

12 (b) Please specify technique: _____

Main constraints	Very high	High	Average	Low	Do not know
Cost of technology	☐	☐	☐	☐	☐

Intellectual property rights	☐	☐	☐	☐	☐
Legal situation	☐	☐	☐	☐	☐
Acceptance of consumers/users	☐	☐	☐	☐	☐
Other (please specify):	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

12 (c) Please specify technique: _____

Main constraints	**Very high**	**High**	**Average**	**Low**	**Do not know**
Cost of technology	☐	☐	☐	☐	☐
Intellectual property rights	☐	☐	☐	☐	☐
Legal situation	☐	☐	☐	☐	☐
Acceptance of consumers/users	☐	☐	☐	☐	☐
Other (please specify):	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

12 (d) Please specify technique: _____

Main constraints	Very high	High	Average	Low	Do not know
Cost of technology	□	□	□	□	□
Intellectual property rights	□	□	□	□	□
Legal situation	□	□	□	□	□
Acceptance of consumers/users	□	□	□	□	□
Other (please specify):	□	□	□	□	□
_____	□	□	□	□	□
_____	□	□	□	□	□
_____	□	□	□	□	□

12 (e) Please specify technique: _____

Main constraints	Very high	High	Average	Low	Do not know
Cost of technology	□	□	□	□	□
Intellectual property rights	□	□	□	□	□
Legal situation	□	□	□	□	□
Lack of acceptance by consumers/users	□	□	□	□	□
Other field (please specify):	□	□	□	□	□
_____	□	□	□	□	□
_____	□	□	□	□	□
_____	□	□	□	□	□

13. If you answered "yes" to the use of "new" plant breeding techniques in table 10, in which areas do you see the major technical and socio economic benefits for development and commercialisation of the new plant breeding techniques? Please assess the relevance of each factor of the following list.

13 (a) Please specify technique: _____

Main benefits	**Very high**	**High**	**Average**	**Low**	**Do not know**
Time factor (compared to conventional breeding)	☐	☐	☐	☐	☐
Better acceptance by consumers/users (compared to transgenesis)	☐	☐	☐	☐	☐
Technological advantage: please specify					☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
Other field (please specify):					
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

13 (b) Please specify technique: _____

Main benefits	**Very high**	**High**	**Average**	**Low**	**Do not know**
Time factor (compared to conventional breeding)	☐	☐	☐	☐	☐
Better acceptance by consumers/users (compared to transgenesis)	☐	☐	☐	☐	☐
Technological advantage: please specify					☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

Other field (please specify):

_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

13 (c) Please specify technique: _____

Main benefits	**Very high**	**High**	**Average**	**Low**	**Do not know**
Time factor (compared to conventional breeding)	☐	☐	☐	☐	☐
Better acceptance by consumers/users (compared to transgenesis)	☐	☐	☐	☐	☐
Technological advantage: please specify					☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

Other field (please specify):

_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

13 (d) Please specify technique: _____

Main benefits	Very high	High	Average	Low	Do not know
Time factor (compared to conventional breeding)	☐	☐	☐	☐	☐
Better acceptance by consumers/users (compared to transgenesis)	☐	☐	☐	☐	☐
Technological advantage: please specify					☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
Other field (please specify):					
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

13 (e) Please specify technique: _____

Main benefits	Very high	High	Average	Low	Do not know
Time factor (compared to conventional breeding)	☐	☐	☐	☐	☐
Better acceptance by consumers/users (compared to transgenesis)	☐	☐	☐	☐	☐
Technological advantage: please specify					☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
Other field (please specify):					
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐
_____	☐	☐	☐	☐	☐

D. COMMENTS OR SUGGESTIONS

►_____
_____
_____
_____
_____
_____

THANK YOU VERY MUCH FOR YOUR CONTRIBUTION!

Privacy statement

The Survey on New Techniques for Plant Breeding is carried out by the New Technologies in Agriculture (Agritech) action of the European Commission's Joint Research Centre (JRC), Institute for Prospective Technological Studies (IPTS). The survey is directed to 50 European companies involved in plant breeding.

The European Union is committed to data protection and privacy as defined in Regulation (EC) No 45/2001. The survey is under the responsibility of the Agritech action leader, Emilio Rodriguez Cerezo, acting as the Controller as defined in the above regulation. The Controller commits himself dealing with the data collected with the necessary confidentiality and security as defined in the regulation on data protection and processes it only for the explicit and legitimate purpose declared and will not further process it in a way incompatible with the purposes. The processing operations are subject to the Notification to the Data Protection Officer (DPO) in accordance with Regulation (EC) 45/2001.

Purpose and data treatment

The purpose of data collection is to establish the analysis of the degree of adoption of new techniques for plant breeding by companies acting in this area. This survey is part of the work program of JRC-IPTS agreed for 2010. The personal data collected and further processed are:

- Company: name, primary sector of activity, home country, company size
- Contact person: name, job title, phone number, e-mail address

The collected personal data and all information related to the above mentioned survey is stored on servers of the JRC-IPTS, the options of which underlie the Commission's security decisions and provisions established by the Directorate of Security for these kind of servers and services. **The information you provide will be treated as confidential and aggregated for the presentation in the report on the project "New plant breeding techniques: Adoption and impact of policy options". The draft final report of this project will be sent to all participants in the survey for comments within a specified deadline.**

Data verification and modification

In case you want to verify the personal data or to have it modified or deleted, please write an e-mail message to the address mentioned under “Contact information”, by specifying your request. Special attention is drawn to the consequences of a delete request, in which case any trace to be able to contact will be lost. Your data is stored as long as follow-up actions to the above mentioned survey are necessary with regard to processing of personal data.

Contact information

In case you have questions related to this survey, or concerning any information processed in the context, or on your rights, feel free to contact the Agritech team, operating under the responsibility of the Controller at the following email address: jrc-ipts-agritech@ec.europa.eu.

Recourse

Complaints, in case of conflict, can be addressed to the European Data Protection Supervisor (EDPS) at www.edps.europa.eu.

ANNEX 14: LITERATURE FINDINGS ON FOOD/FEED AND ENVIRONMENTAL SAFETY AND ON REGULATORY ISSUES[57]

Zinc Finger Nuclease Technique (ZFN)	Issues covered			Main conclusions, discussion or remarks
	Food and feed safety	Safety for the environment	Regulatory issues	
Wright et al. (2005)			X	Preliminary discussion.

Oligonucleotide directed mutagenesis (ODM)	Issues covered			Main conclusions, discussion or remarks
	Food and feed safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The COGEM 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 & Visser (2009)	X	X		Schaart & Visser (2009) conclude that plants that are obtained through ODM have a single change in the target gene that could also be obtained through mutation breeding. Supposing that mutated plants are free of incorporated oligonucleotides, the consequences for food and feed safety are similar to those of the baseline (i.e. plants generated by mutation breeding through irradiation or chemicals). Like those plants, crops obtained through ODM should be tested for undesired traits before being released.
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	Preliminary discussion.

57 It is noted that the presented information and conclusions summarise the opinion of the authors of the publications, but do not necessarily reflect the opinion of the authors of the JRC report.

Cisgenesis and Intragenesis	Issues covered			Main conclusions, discussion or remarks
	Food and feed safety	Safety for the environment	Regulatory issues	
Schaart & Visser (2009)	X	X		Concerning the consequences for food and feed safety and the environmental consequences of cisgenesis, Schaart & 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, Schaart & Visser (2009) conclude that no general statement concerning 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 of native genes, the intragenic plants may be comparable to plants with knock-out mutations. Such plants can be used as baseline.
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 the plant's 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).
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 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).
Jacobsen & Schaart (2009)	X			Jacobsen & Schaart (2009) evaluate the biosafety of T-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 small compared to the risks of conventional breeding and mutation breeding which are regarded as suitable baseline for the risk assessment of transgenic plants.
Russell & Sparrow (2008)	X	X	X	Russell & Sparrow (2008) compare the regulatory system in the USA, 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.
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.
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".
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 for organic farming because of ethical reasons (respect of naturalness and integrity of all organisms).
Jacobsen & Schouten (2007)			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 contain only 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.
Jacobsen & Schouten (2008)	X	X	X	
Jacobsen & Schouten (2009)	X		X	
Schouten & Jacobsen (2008)			X	
Schouten et al. (2006a)	X	X	X	
Schouten et al. (2006b)	X	X	X	
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.
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.

RNA-dependent DNA methylation (RdDM)	**Issues covered**			**Main conclusions, discussion or remarks**
	Food and feed safety	**Safety for the environment**	**Regulatory issues**	
COGEM (2006b)	X	X	X	The COGEM report observes that it is too early to date to make judgements on any environmental risks of epigenetic mutants as too little is known concerning 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.

Grafting (on GM rootstock)	Issues covered			Main conclusions, discussion or remarks
	Food and feed safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The COGEM report points out that it has to be considered if a graft is in a legal sense two different plants or one plant. The authors 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.
Schaart & Visser (2009)	X	X		Schaart & Visser (2009) note 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 on the scion and that 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.
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 the determination of isolation distances.
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.
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.

Reverse breeding	Issues covered			Main conclusions, discussion or remarks
	Food and feed safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The authors of the COGEM 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 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 authors however recommend 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 (2009) conclude that food and feed of crops obtained through reverse breeding are as safe as products from the original heterozygous lines, because 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.
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 for organic farming because of ethical reasons (respect of naturalness and integrity of all organisms).

Agro-infiltration	Issues covered			Main conclusions, discussion or remarks
	Food and feed safety	Safety for the environment	Regulatory issues	
COGEM (2006b)	X	X	X	The COGEM report concludes that plants obtained through agro-infiltration (COGEM used the term agro-inoculation for this technique at that time) should, in principle, be considered as not transgenic. They note however that literature data on the (im)possibility of unintended transformation of offspring and contamination of seed with A. tumefaciens as a result of the use of the technique is absent. However, the authors recommend assigning a GMO-free status to offspring of plants that have undergone an agro-inoculation treatment, if it can be ruled out that A. tumefaciens gets into the offspring.
Schaart & Visser (2009)	X	X		The report Schaart & Visser (2009) recommends proving that crop plants originating from plants which are selected using agro-infiltration are free of Agrobacterium, Agrobacterium chromosomal DNA and binary vector sequences before they are released into the environment. If agro-infiltration is used for gene-silencing, the silencing effect may still be present in non-GM offspring. Therefore, it should be evaluated if the expression of the genes that have been silenced falls within the bandwidth of expression of a suitable baseline. If the absence of Agrobacterium, Agrobacterium chromosomal DNA and binary vector sequences and (where applicable) of gene silencing has been proven, the authors foresee no environmental consequences when this material is released into the environment and food and feed safety would be comparable to products from the original plants before agro-infiltration.

ANNEX 15: WORKING GROUP ON THE CHANGES IN THE PLANT GENOME - REPORT

EVALUATION OF CHANGES IN THE GENOME IN PLANTS THROUGH APPLICATION OF NEW PLANT BREEDING TECHNIQUES

IN THE FRAMEWORK OF THE PROJECT "NEW PLANT BREEDING TECHNIQUES: ADOPTION AND ECONOMIC IMPACT"

REPORT
30-09-2010

Dr Boet Glandorf
National Institute of Public Health and the Environment
Bilthoven, the Netherlands

Prof. Marc de Loose
Institute for Agricultural and Fisheries Research (ILVO)
Merelbeke, Belgium

Prof. Howard Davies
Scottish Crop Research Institute
Dundee, UK

1. Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

Definition

Three applications of Zinc Finger Nucleases (ZFNs) are recognised. These are designated as ZFN-1, ZFN-2 and ZFN-3.

ZFN-1

Genes encoding ZFNs are delivered to plant cells without a repair template. The ZFN binds to the DNA and generates a site-specific double strand break (DSB). The natural DNA-repair process which occurs through non-homologous end-joining (NHEJ) leads to site-specific random mutations leading to changes to one or a few base pairs, or to short deletions or insertions.

ZFN-2

Genes encoding ZFNs are delivered to plant cells along with a short repair template. The ZFN binds to the DNA and generates a site-specific DSB. Gene repair mechanisms generate site-specific point mutations e.g. changes to one or a few base pairs, through homologous recombination (HR).

ZFN-3

Genes encoding ZFNs are delivered to plant cells along with a large stretch of DNA (several kbp (kilo base pairs)), the ends of which are homologous to the DNA sequences flanking the cleavage site. As a result, the DNA stretch is inserted in the genome in a site-specific manner.

Rationale for use in plant breeding

The rationale for using the ZFN approach is to create site-specific mutations (targeted mutations) or gene inactivation (in the case of the ZFN-1 and ZFN-2 techniques). The ZFN-3 approach can be used for targeted gene addition, gene replacement and trait stacking. Specific gene targeting can prevent so-called "position effects" caused by random insertion of genes in the genome.

The genes coding for the ZFN complex can be introduced into the cells by transformation using viral vectors encoding the ZFN protein complex, A. *tumefaciens*-mediated transformation, or particle bombardment. ZFNs are typically expressed transiently from a vector (plasmid, virus). However, in the future they may be delivered directly as proteins.

Mechanism

ZFNs are proteins which are custom-designed to cut DNA at specific sequences. They consist of a "zinc finger", a DNA-binding domain that recognises specific 3 bp DNA sequences, and an effector protein which is usually the nuclease *Fok*I. *Fok*I is a bacterial type IIS restriction endonuclease that recognises 5'-GGATG-3': 5'-CATCC-3' in duplex DNA and cleaves 9/13 nucleotides (nt) downstream of the recognition site (Durai et al., 2005). ZFN function in pairs, each recognizing the opposite DNA strand, thereby forming a ZFN complex. Two ZFNs can therefore create a DSB at a specific site in the DNA. The DSB created by ZFNs stimulates the cell's repair mechanism, the process of HR, and insertion of DNA fragments. In general three "zinc fingers" are used, which makes it possible to recognise DNA sequences of 9 bp as monomer and 18 bp as dimer.

Intended changes/effects

ZFN-1

With the ZFN-1 approach, no repair template is provided to the cells together with the ZFN proteins. The DSB is corrected by NHEJ, which is a natural DNA repair system in the cell. This often results in substitutions to one or only a few bases or in small localised deletions or insertions. The ZFN-1 technique has been used as an efficient mutagenesis method in *Arabidopsis*, tobacco and maize (Lloyd et al., 2005; Maeder et al., 2008; Shukla et al., 2009; Tovkach et al., 2009). De Pater et al. (2009) reported mutation frequencies of 2% in *Arabidopsis* after introducing ZFNs in the genome using *Agrobacterium tumefaciens* floral dip transformation. Mutation frequencies of 40% were observed in tobacco when *SuRA* and *SuRB* genes were targeted with specific ZFNs (Townsend et al., 2009). These genes code for mutated tobacco acetolactate synthase conferring resistance to specific herbicides. 2% of the herbicide resistant plants demonstrated mutations as far as 1.3 kbp from the ZFN cleavage site. In *Arabidopsis* a ZFN construct under the control of a heat shock protein resulted in 78% deletions of between 1 to 52 bp and 13% insertions of between 1 to 4 bp. 8% of deletions were accompanied by insertions (Lloyd et al., 2005). In 10% of the individuals that contained ZFN-induced mutations, mutants were present in the next generation. Should these mutations occur in a coding region, it is calculated that 77% of the mutations would produce a frame shift, 14% would delete between one to four amino acids, 7% would delete eight or more amino acids and 2% would result in changes in amino acids, thereby resulting in a high frequency of functional gene knock-outs. This observation is similar to findings in most other studies and actual frequencies are probably higher (Lloyd et al., 2005).

ZFN-2

With the ZFN-2 approach, a continuous stretch of DNA is delivered to the cell simultaneously with the ZFN. This template DNA is homologous to the targeted area, spanning a few kbp, and overlaps the region of the DSB. The template DNA contains the specific base pair alterations to be introduced in the genome by HR, which occurs at a very low rate in plants compared to NHEJ. Estimates of HR in tobacco range from one HR event per 8.4 $x10^5$ to 2.2 $x\ 10^6$ illegitimate events (Wright et al., 2005). These authors demonstrated that chromosome breaks created by ZFNs enhance the frequency of localised HR by a factor 10^4 to 10^6, resulting in more than one HR for every 10 illegitimate recombination events. The frequency of HR was measured by restoring the function of a defective GUS:NPTII (beta-glucuronidase, neomycin phosphotransferase) reporter gene integrated at various chromosomal sites in 10 different tobacco lines. Twenty per cent of the reporter system genes were repaired solely by HR whereas the remainder had associated DNA insertions or deletions consistent with repair by both HR and NHEJ. No difference was observed between the chromosomal locations. Fidelity of gene targeting was approximately 20%, with 20% of the characterised gene targeting events being free from any DNA insertions or deletions sustained during the repair of the target locus.

ZFN-3

With the ZFN-3 approach a recombinant DNA molecule is constructed in which the DNA fragment of the gene cassette of interest is sandwiched between stretches of DNA that are homologous with the DNA sequences flanking the DSB site. This DNA construct, together with the ZFN, is delivered to the cell. Highly efficiency targeting of DNA to an endogenous genomic locus in the cell can be obtained by HR. Targeted transgene integration using the ZFN technique has been demonstrated in tobacco (Cai et al., 2009), maize (Shukla et al., 2009) and *Arabidopsis* (Tzfira and White, 2005). Incoming DNA can be targeted to a relatively large region surrounding the DSB (de Pater et al., 2009).

ZFN-1 to -3

When considering the genomic changes that can be induced for all ZFN approaches, the question is which generation of plants should be considered. If ZFNs are expressed from a vector, the ZFN genes are intended to be present transiently in the cell and are expected to be absent from the final product that will be commercialised. ZFN genes can also be integrated in the plant genome as a transgenic construct. In this case the transgenic ZFN construct would be inherited. Offspring that still carry the ZFN construct would have to be selected out.

Therefore, only changes in the genome of the final product not related to the presence of ZFN genes are considered. A screening procedure to test for the absence of the ZFN genes would be a logical part of the selection process.

Unintended changes/effects

ZFNs do not always have the desired sequence specificity and affinity because not all of the ZFNs designed and available bind to their cognate DNA triplets in a highly sequence-specific manner. They also bind to sites with degenerate sequences (Durai et al., 2005). This non-specific binding can lead to non-specific DSBs, resulting in unintended mutations at such a high level that human cell cytotoxicity occurs (Wu et al., 2007). Four-finger ZFNs that recognise 24 bp DNA sequences have been shown to promote highly sequence-specific cleavage in human cells, while exhibiting decreased cytotoxicity (Urnov et al., 2005). It is therefore hypothesised that four-finger ZFNs would increase specificity compared to three-finger ZFNs. Furthermore, sustained expression of ZFNs is likely to contribute to cellular toxicity due to non-specific binding leading to unwanted DSBs in the genome (Porteus and Carroll, 2005). Inducible promoters could be used to control this problem.

The literature indicates that, given the current state-of-art of the technology, non-specific mutations resulting from non-specific binding of the ZFNs are likely to occur.

Baseline/safety issues

Changes in the genome induced by the ZFN-1 and ZFN-2 techniques can be compared to changes that could occur from natural mechanisms which operate during plant breeding, or from those induced by breeding techniques such as mutagenesis using irradiation or chemical mutagens. The difference is that changes induced by ZFN-1 and ZFN-2 techniques are intended to be site-specific. To date, it is not clear how well this technique works in practice and to what extent off-target effects occur due to non-specific breaks. A point to consider for safety is that with ZFN multiple subsequent site-specific changes may be induced in a single organism, which is not possible by chemical or natural means. Genomic changes produced by the ZFN 3 approach are comparable to those occurring as a consequence of transgenesis. However, since the gene(s) can be targeted to a specific site in the genome, unexpected effects due to so-called 'position effects' are expected to be less in comparison to genetic modification.

2. Oligonucleotide directed mutagenesis (ODM)

Definition

Also known as Targeted Gene Repair, Oligonucleotide-directed Gene Targeting, Genoplasty, Chimeraplasty, etc.

Oligonucleotides target homologous DNA and induce site-specific nucleotide substitutions, insertions or deletions through repair mechanisms. The following types of oligonucletides are used: Single stranded DNA oligonucleotides, chimeric oligonucleotides, triple helix-forming oligonucleotides (TFOs) and RNA oligonucleotides.

Rationale for use in plant breeding

ODM provides a method to introduce specific mutations in specific genes or DNA sequences in plants (Breyer et al., 2009). These changes may result in:

1. modified amino acid sequences of proteins;
2. complete gene knockouts by introducing stop codons or frameshift; mutations and
3. modified gene expression by making changes in promoter sequences.

Such mutations may be useful to inhibit unwanted gene expression, to increase beneficial gene expression or to produce changes in proteins resulting in more efficient and effective molecules e.g. enzymes.

ODM can be used in plant breeding to create genetic variation by introducing specific mutations leading to the desired phenotype. The induction of gene-targeted mutation using oligonucleotides has already been performed in agriculturally important plants including maize, tobacco, rice, wheat and tomato (e.g. to introduce resistance to sulfonylurea herbicides (Breyer et al., 2009)). With the use of efficient screening methods other objectives will become possible, including mutants with increased abiotic stress tolerance, increased insect or virus resistance and increased yield.

Some major drawbacks have been observed in the application for plant breeding purposes e.g., the spontaneous occurrence of somatic mutations which obscure the mutation of interest (Ruiter et al., 2003), the low frequency of the repair event (Li et al., 2007) and difficulty in further selection and regeneration of plants containing the mutation due to the absence of a selective marker. However, by using efficient DNA-based screening methods identification of the plants with the desired mutation is becoming feasible.

Mechanism

ODM employs oligonucleotides for targeted (site-specific) changes of one or a few adjacent nucleotides. Oligonucleotides of approximately 20 to 100 nt (nucleotides) are delivered to the cells by methods such as electroporation, polyethylene glycol (PEG)-mediated transfection and natural transformation. The technique exploits the sequence specific interaction of the oligonucleotide with the resident DNA of the cells resulting in gene targeting. This directs the proposed genetic modification to a specific region in the DNA or even to a specific base pair. Changes can include the introduction of a new mutation (replacement of one or a few base pairs or introduction of short deletions), or reversion of an existing mutation which may lead to changes in the expression of a gene. Four different types of oligonucleotides have been used so far:

1. single-stranded homologous DNA with a single mismatch to the target sequence (Campbell et al., 1989);
2. chimeric oligonucleotides consisting of RNA stretches within single-stranded DNA (Beetham et al., 1999);
3. triple helix-forming oligonucleotides (TFOs) which form relatively stable associations with duplex DNA via Hoogsteen hydrogen bonds (Simon et al., 2008);
4. RNA oligonucleotides to induce RNA-mediated targeted DNA nucleotide sequence changes and RNA-templated DNA repair resulting in point mutations (Storici, 2008).

Details on the mechanisms involved in ODM-induced DNA sequence changes are not completely understood at present although evidence has been provided that the type of oligonucleotide, the status of the resident DNA and its enclosure in the chromatin structure, the components of the cellular DNA recombination and repair machinery, affect the outcome of the targeted DNA sequence change (Dong et al., 2006).

Intended changes/effects

If the oligonucleotide and the experimental protocol are adequately designed, the mutation induced by ODM should be highly specific. Organisms developed through ODM cannot be distinguished at the molecular level from organisms bearing the same mutation obtained through mutation techniques such as irradiation or chemical mutagenesis or through selection from natural populations.

Unintended changes/effects

The development of organisms using ODM technology is expected to generate fewer unintentional changes or effects than those generated by breeding techniques based on irradiation or chemical mutagenesis. An advantage of this technology is that it does not use integrative vectors and thus eliminates the risk of any associated insertional mutagenesis. It also acts on specific genes and does not introduce foreign DNA sequences into the target genome (Reiss, 2003). However, the application of an ODM approach does not exclude spontaneous mutations randomly in the genome (Ruiter et al., 2003). With the current molecular approaches it is feasible to test for the changes obtained by the mutagenesis in the target locus but it is much more difficult to identify potentially induced mutations at non-target loci.

Baseline/safety issues

ODM does not result in other changes in the genome compared with mutations that occur as a result of natural processes or via irradiation and chemically induced approaches. Potential safety issues may be related to changes in the expression of endogenous genes or to a specific change in the amino acid sequence of an endogenous protein.

3. Cisgenesis and intragenesis

Definition

A DNA fragment from the plant species itself or from a cross-compatible plant species is inserted into the plant genome. In the case of cisgenesis, the inserted gene is unchanged and includes its own introns and regulatory sequences. 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.

Rationale for use in plant breeding

The uses are the same as for transgenic approaches i.e. the introduction of new traits or modifications to existing traits to add value to existing germplasm without the potential problems of linkage drag associated with conventional crossing. Changes introduced could include improved resistance to biotic and abiotic stresses, improved quality and nutritional value etc. Conventional crossing can be used to introgress traits introduced using cisgenic/intragenic into other cultivars and also to combine (stack) multiple traits where required. As intragenics can use constructs which contain new combinations of genes and regulatory sequences, including the use of antisense or RNAi (RNA interference), it provides scope to modify traits in a way that cisgenics could not.

A major rationale for using these approaches in plant breeding is the issue of consumer acceptance and the argument that the use of DNA from within cross-compatible species (mimicking the potential end products of traditional breeding) is a safer option than transgenesis. There is reasonable evidence that consumers are more comfortable with the use of genes from within the same species than transgenes originating from organisms such as bacteria (Schouten et al., 2006a; Rommens, 2010). However, the definition of a species and what "cross-compatible" means needs to be considered as fairly wide crosses are possible with or without intervention approaches such as hybrid rescue.

Mechanism

Cisgenics and intragenics plants are produced by the same transformation techniques as transgenic plants e.g. *Agrobacterium*-mediated transformation (Belfanti et al., 2004), following the isolation of genes from the host. In theory, biolistics could also be used. With *Agrobacterium*-mediated transformation the vectors used may contain *Agrobacterium* T-DNA (transfer DNA) border sequences to facilitate the insertion of the target genes into the plant genome. However, specific vectors have been constructed for cisgenic/intragenic approaches which use DNA sequences originating from the same crop species or related species to insert the target genes. These sequences have sufficient homology with *Agrobacterium* T-DNA sequences to allow this function. This approach is termed the P(plant)-DNA approach (Rommens et al., 2004; Conner et al., 2007). The general presence of such P-DNA within the genomes of plants remains to be established. The P-DNA strategy may often require relaxing the sequence similarity to authentic T-DNA borders (Conner et al., 2007).

Agrobacterium cleavage and secretion enzymes release the P-DNA from a binary vector for processing and transfer to plant cell nuclei. Upon transfer, the P-DNA integrates into double-stranded chromosome breaks (Rommens, 2007). Genes (single, multiple) and regulatory elements will be incorporated into the genome (e.g. the nuclear genome) and inherited as stable events in the expected manner.

Intended changes/effects

The intended changes relate to modifying the expression of target genes through stable integration to the host genome, as is the case for transgenesis. The intended changes are driven by prior knowledge of the function of the genes whose expression is modified using the cisgenic/intragenic approach. Cisgenic/intragenic plants might contain some small, non-coding bacterial sequences from the vector such as T-DNA borders. Where P-DNA approaches are used, bacterial DNA is absent.

Unintended changes/effects

Irrespective of whether the cisgenic or intragenic approaches are used there is the possibility that the inserts interrupt open reading frames (ORFs) in the host plant or create new ones as a consequence of the insertion process. Deletion of host DNA can also occur following insertion. This could give rise to unintended effects. The same issues are identified as a possible risk for transgenics.

Cisgenic constructs will contain genes and regulatory elements in their "natural" state. Thus similar products could be produced using conventional breeding approaches (Schouten et al., 2006a; Jacobsen and Schouten, 2009). However the transfer of such endogenous genes and regulatory elements to another plant could result in modified levels of expression of the target gene(s) and even gene silencing. As intragenics uses new combinations of genes and regulatory sequences, gene expression may be changed more extensively (spatially and quantitatively) than with cisgenics. Furthermore, as intragenic approaches also use RNAi for gene silencing the possibility of effects on other genes and metabolic pathways cannot be excluded.

Baseline/safety issues

The possibility exists that inserts interrupt known ORFs or create new ones as a consequence of the insertion process. Deletion of host DNA can also occur following insertion. Conventional breeding can also result in disruptions to ORFs and other molecular changes including deletions and recombinations. The same can be said for mutation breeding and variation induced by somaclonal variation.

It has been argued that cisgenesis may be safer than conventional breeding because it prevents introduction of genes via linkage drag which could lead to unwanted traits (e.g., increase glycoalkaloid content to a higher level than allowed in the regulations for breeder's rights (Haverkort et al., 2008)). However, the issue of any silencing of endogenous genes needs to be considered.

The cisgenic/intragenic approach is based on the assumption of cross-compatibility of the host plant and the plant used to provide the genes. In some cases it could be argued that the germplasm used to source the genes (e.g. a distal wild relative of the recipient plant) may not have a history of safe use in the food chain but this would only be relevant on case-by-case basis depending on the genes used.

Given that cisgenic/intragenic organisms may contain new proteins, or greatly altered levels of familiar proteins, it has been argued that they generate similar concerns about safety as transgenic organisms (Russell and Sparrow, 2008 and references therein).

Intragenics offer considerably more options for modifying gene expression and trait development than cisgenics since genes and their promoters and regulatory elements are interchangeable. Intragenics can also include silencing mechanisms e.g. RNAi using within species DNA sequences (Rommens, 2007; Rommens et al., 2007; Rommens et al., 2008). There is therefore the potential for more unintended effects than with cisgenics.

4. RNA-dependent DNA methylation (RdDM).

Definition

Genes encoding RNAs which are homologous to plant sequences, like promoter regions, are delivered to the plant cells. These genes, once transcribed, give rise to the formation of small double stranded RNAs (dsRNAs). They induce methylation of the homologous sequences and consequently inhibit their transcription.

Rationale for use in plant breeding

The rationale for the use of RNA-dependent DNA methylation (RdDM) is the silencing of specific genes in plants, without causing DNA mutations. RdDM can be used in plant breeding to silence specific genes by the introduction of inverted repeat (IR) sequences and other transgenes that provide template RNAs that are converted into dsRNAs. These dsRNAs lead to methylation of the promoter of the gene(s) to be silenced. The dsRNA triggering promoter methylation can be introduced into the plant by transfection and can be synthesised *in vivo* from a heterozygous recombinant gene (RNAi insert) or by using a vector system (e.g. plasmid) carrying the RNAi insert. In the following plant generation individuals which do not contain the RNAi insert, but which retain the methylated promoter and the target trait, are selected from the segregants. In this way, modified organisms can be obtained with specific genes silenced but without the RNAi insert in the genome. Breeding objectives achieved by silencing of genes in plants are for example to obtain male sterility in maize by silencing of the fertility gene Ms45 (Cigan et al., 2005) or to reduce the amylose content in potatoes by silencing the GBSS (Granule-bound starch synthase) gene (Heiligersig et al., 2006).

Mechanism

RdDM is one of several RNA interference (RNAi)-mediated pathways in the nucleus and uses small RNAs (21-24 nt) to methylate sequences in the plant, thereby leading to gene silencing. RdDM is induced by dsRNA created by the "dicer" class of ribonucleases and, in concert with numerous proteins, leads to *de novo* cytosine methylation at symmetric CpG/CpHpG and asymmetric CpHpH sites (where H=A, T or G (Matzke et al., 2004)).

Several reviews describe the mechanism of RdDM and the components involved (see for example Wassenegger, 2000; Vaucheret and Fagard, 2001; Pickford and Cogoni, 2003; Matzke et al., 2004; Huettel et al., 2007; Lavrov and Kibanov, 2007; Shiba and Takayania, 2007; Eamens et al., 2008; Chinnusamy and Zhu, 2009; Chen, 2010). RdDM is proposed to play a role in stress responses, plant development (Huettel et al., 2007) and in plant defence (Mette et al., 2000).

Intended changes/effects

Introduced sequences can give rise to non-coding RNAs (ncRNAs) such as small interfering RNA (siRNA) or microRNA (miRNA). siRNAs are processed from long, perfectly dsRNA and miRNAs from single-stranded RNA transcripts (transcribed from miRNA genes) that have the ability to fold back onto themselves to produce imperfectly double-stranded stem loop precursor structures (Eamens et al., 2008). Inverted Repeat (IR) constructs seem to be the most effective (Mette et al., 2000; Muskens et al., 2000). If the dsRNA formed is homologous to promoter sequences, the promoter may be methylated and the downstream gene silenced. A minimum of ca. 30 bp of homologous sequence is necessary for methylation (Matzke et al., 2004).

Silencing of genes using this approach has been reported for several plant species, including *Arabidopsis*, tobacco, maize, *Petunia* and *Pinus*. The efficiency of silencing can be up to 90% (Eamens et al., 2008) and is dependent on the active transcription of the promoter (Lavrov and Kibanov, 2007). Generally, the degree of silencing is related to the degree of methylation (Fischer et al., 2008), but this is not always the case (Okano et al., 2008). The amount of silencing in the F1 generation can vary by more than a hundred-fold and these differences between individuals can become more prominent in progressive generations (Fischer et al., 2008). Silencing, and differences in silencing, have been observed to be transmitted to at least the F3 generation.

Promoters of endogenous genes appear to be less amenable to silencing than transgene promoters. Cytosine content and local DNA features have been proposed as factors affecting RdDM in plants (Fischer et al., 2008; Okano et al., 2008). Both constitutive and tissue-specific plant promoters are capable of being transcriptionally repressed (Cigan et al., 2005). Methylation is restricted to the region of sequence homology with the dsRNA. No spreading of methylation into sequences flanking the region of homology between the IR RNA (also known as hairpin RNA (hpRNA)) and the target DNA has been observed (Fu et al., 2000; Kunz et al., 2003; Dalakouras et al., 2009).

When the template RNA for dsRNA is introduced by transfection or by a vector system, the templates are intended to be present only transiently in the cell and are expected to be absent from the final commercialised product. When an RNAi construct is used, commercial products lacking the construct can be obtained by segregation. In all cases a screening procedure to test for the absence of this construct would be a logical part of the selection process. Therefore, only changes in the genome of the final product in the absence of the RNA template are considered in this document.

Unintended changes/effects

It is not clear for how many generations the effect of gene silencing by RdDM remains in the absence of the inducing construct. An unintended effect could therefore be the loss of silencing of the specific gene in the commercial product. Another potential unintended effect could be the silencing of genes with homologous promoter sequences. Alternatively, the production of other small RNAs from an hpRNA can occur that may regulate the expression of other genes not intended to be manipulated (Chen, 2010).

Baseline/safety issues

RdDM is not expected to cause changes in the genome other than DNA methylation. Methylation of DNA is a natural phenomenon and can be induced by environmental conditions and by traditional breeding. This is illustrated by the fact that methylation is widespread in plant chromosomes. Indeed, ca. 20% of the *Arabidopsis* genome is methylated (Shiba and Takayania, 2007). Potential safety issues may therefore only be related to changes in the expression levels of targeted endogenous genes.

5. Grafting (on GM rootstock)

Definition

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 regard to plant breeding the grafting of a non-GM scion onto a GM rootstock is considered to be the main approach. However, it is clearly possible to graft a GM scion onto a non-GM root stock and indeed a GM scion onto a genetically modified rootstock.

Rationale for use in plant breeding

Grafting combines the desired properties of a rootstock with those of the donor scion. There are many potential benefits from the use of GM rootstocks in grafting including enhanced root performance (disease resistance, root growth, nutrient and water acquisition) which in turn enhances the performance of the scion resulting in increased yield and quality.

Mechanism

GM rootstocks can be isolated from transformed plants developed using standard approaches including *Agrobacterium* and biolistics-mediated gene transfer. The GM rootstock is then used for grafting onto the desired scion. For successful grafting to take place, the vascular systems of the root and shoot need to be connected to allow the flow of water, nutrients, assimilates and macro molecules between the various plant parts.

Intended changes/effects

Should both the rootstock and scion be transformed using methods known to modify the genome then the entire plant is considered to be GM. Should a GM scion be grafted onto a non-GM rootstock then clearly above ground parts such as seeds, edible components, etc. will be transgenic. If only the rootstock is transformed then intended changes to the genome are targeted to root tissues.

Intended changes will be dictated by the selection of promoters and gene sequences which are targeted for modified expression, as would be the case for a "standard" transgenic plant. However, it is conceivable that there might be an intention to transform only the rootstock with a view to changing protein or gene expression in the scion due to the movement of specific proteins and/or RNA from the roots to the scion. In this way a GM rootstock could be used to introduce new traits into a range of genetically distinct scions.

Unintended changes/effects

One consideration is whether or not mechanisms exist for the transmission of nucleic acids, proteins or other metabolites which could induce changes to the genome in the non-transformed tissues following grafting. With respect to the possible movement of DNA between rootstock and scion which could result in genome changes in the scion there is little evidence that this is an issue. Stegemann and Bock (2009)

have reported the transfer of plastid genetic information in a graft from rootstock cells to the cells of the scion and vice versa. Chimeric cells were recovered from the graft site but it was not clear if the genetic information was transferred as DNA fragments, as entire plastid genome or as plastid. Genetic exchange appeared to be restricted to graft sites only (flowers and fruits from a non-GM scion did not contain GM DNA sequences from the GM rootstock). One should be able to conclude that unintended changes to the coding sequence of a non-GM scion grafted onto a GM rootstock do not occur.

With regard to unintended effects resulting from the transmission of other macromolecules from root to scion, it is known that recombinant proteins, hormones and non coding RNA (e.g. siRNAs) can be transported from the GM rootstock of a graft to the scion where they can induce an effect. It is known that RNAi can lead to RNA-directed DNA methylation of promoter regions, resulting on modified expression of the target genes (see Section 4). So, although the resulting offspring from a graft can be regarded as non-GM, mitotically and meiotically heritable (epigenetic) changes in gene expression that do not involve a change in the DNA sequence can still occur (Martienssen and Colot, 2001).

Baseline/safety issues

The major issue relates to any unintended changes in gene, protein and trait expression in the scion resulting from unwanted movement of proteins and RNA from GM roots to non-GM scions.

6. Reverse breeding

Definition

Homozygous parental lines are produced from selected heterozygous plants by suppressing meiotic recombination. This suppression is obtained through RNAi-mediate down-regulation of genes involved in the meiotic recombination process. Subsequently, double haploid (DH) homozygous lines are produced and hybridised, in order to reconstitute the original genetic composition of the selected heterozygous plants.

Rationale for use in plant breeding

The rationale for the use of reverse breeding is to obtain homozygous parental lines for the production of F1 hybrids with a high level of heterosis in a much shorter timeframe than conventional breeding. Furthermore, it provides more flexibility in combining desired traits in a heterozygous setting. Double haploid (DH) plants are screened for the absence of the RNAi construct before they are crossed to the complementary parent to obtain the hybrid variety. The hybrid variety is the final commercial product. Screening for the absence of the RNAi construct during the breeding process is therefore taken as a requirement. Therefore, only changes in the genome of the final product in the absence of the RNAi construct are considered in this document.

Mechanism

To obtain the homozygous parental lines from the F1 hybrid, meiotic recombination is suppressed in the selected heterozygous line through RNAi-mediated down-regulation of genes, such as *dmc1* and *spo11*, which are involved in the meiotic recombination process. This will lead to haploid microspores (immature pollen grain) from which the genome will subsequently be doubled. The diploid microspores will eventually be developed into embryos and subsequently into homozygous plants using tissue culture techniques.

Intended changes/effects

The intended goal of the technique is to generate perfectly complementing homozygous parental lines through a suppression of meiotic crossovers and the subsequent fixation of non-recombinant chromosomes in homozygous DH lines (Dirks et al., 2009). In this respect, there are no changes foreseen in the genome of the selected non-GM offspring.

Unintended changes/effects

To date there are very few publications on reverse breeding. Therefore, few data are availabe on unintended changes in the genome. Unintended effects could include the silencing of other homologous sequences in the genome as a result of the presence of the RNAi construct. This would not induce genomic changes, but could affect expression levels. Another unintended effect of the technique could be an incomplete suppression of meiosis. This would lead to some degree of meiosis and recombination, which are natural processes in plants.

Baseline/Safety issues

Silencing of other homologous sequences in the genome by the RNAi construct could affect expression levels, which can also occur under natural conditions. Suppression of meiosis, incomplete or not, can also be obtained by chemical and physical means or by environmental factors (Patent: Dirks et al., 2003).

7. Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

Definition

Plant tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium sp.* containing a genetic construct. In most of the cases these technologies are carried out on vegetative plant tissues, especially young leaves. The genetic construct is locally expressed at a high level during the first few days after the infiltration, without being integrated into the plant genome. An exception is floral dip transformation where flowering plants are infiltrated with *Agrobacterium* to obtain transformed seeds. Related methods in this context are agro-infection and agro-inoculation.

Rationale for use in plant breeding

In agro-infiltration *Agrobacterium* is used to introduce large numbers of copies of foreign DNA into the plant cells where they are used as templates for the transcription/translation machinery. As a result, gene and protein expression generally exceed that in transgenic plants in which the same construct is stably integrated (Sainsbury and Lomonossoff, 2008). This approach can be used for transient expression to study the functionality of a gene construct (De Paepe et al., 2009) or to produce a particular protein within the area of the leaf infiltrated in order to study its biological activity (Vleeshouwers et al., 2006).

Transient expression of gene constructs is frequently used in a research and development context: e.g. to study the functionality and or the interaction of gene products within plant cells, to evaluate the impact of gene knock-outs, to simulate specific aspects of plant pathogen interactions, and to analyse the functionality of regulatory elements in gene constructs. The advantage is that in a short time period several variables can be studied. It facilitates the identification of genes or sequences within a gene that can then be deployed to develop transgenic plants with target genetic elements stably integrated. It is also used to select plant genotypes with the desired biological response to the presence of particular genes or gene products e.g. selecting plants with the desired pathogen response (Cruz et al., 1999).

In this case agro-infiltration is a screening tool carried out on detached plant parts or on intact plants. After the observations in many cases the infiltrated plants will be destroyed and plants which are genetically identical may be used as parents for further breeding. But in case the progeny of the infiltrated plant is used for further breeding, the seeds will not be transgenic as no genes are inserted into the genome.

Transient expression has also been developed as a production platform for high value recombinant proteins. The approach can result in a high yield of the end product. In all cases, the plant of interest is the agro-infiltrated plant and not the progeny (Pogue et al., 2010).

Mechanism

Depending on the tissues and the type of constructs infiltrated, three types of agro-infiltration can be distinguished:

1. *"Agro-infiltration sensu stricto":*

Non-germline tissues are infiltrated with non-replicative constructs in order to obtain localised expression in the infiltrated area. The infiltration can be carried out on both attached and detached plant parts (Manavella and Chan, 2009). In the case of detached plant parts the experiments are often carried out in tissue culture conditions. In some cases e.g. where there is a long latency period for the effect under study, it is necessary to work directly with whole plants and to rescue the plants with the interesting phenotype.

2. *"Agro-inoculation" or "agro-infection":*

Non-germline tissues (typically leaf tissues) are infiltrated with a construct containing the foreign gene in a full-length virus vector to facilitate spreading and expression of the target gene in the entire plant (Vleeshouwers et al., 2006).

3. *"Floral dip":*

Germline tissues (typically flowers) are infiltrated with *Agrobacterium* containing a T-DNA construct to stably transform the female gametocyte and obtain GM seeds for further study. GM plants derived from this approach do not differ from GM plants obtained by other transformation methods.

Intended changes/effects

The intended goal of the technique is the temporary expression of specific coding sequences without integration of the introduced DNA in the plant genome. However, in the case of the floral dip it is the aim to obtain stably transformed seedlings without the need for a plant cell regeneration phase. The resulting plant has the same properties as a transgenic plant.

Unintended changes/effects

The aim is the transient and temporary expression of a coding sequence as such or to study the biological response of the plant cells or plants to the expressed genes. However, integration of T-DNA fragments into the genome of cells in the infiltrated area cannot be excluded. This is true for agro-infiltration and for agro-inoculation/agro-infection. In the case of agro-inoculation/agro-infection, the spreading of the gene construct introduced into the viral genome is caused by systemic spreading of RNA viruses throughout the plant via plasmodesmata. Since the gene construct are spread via RNA molecules, they do not integrate into the plant genome.

Baseline/safety issues

Agro-infiltration is used to screen for genotypes with valuable phenotypes that can then be used in breeding programmes. For instance, agro-infiltration with specific genes from pathogens can be used to evaluate plant resistance and the mechanisms underpinning the resistance. The most resistant plant identified from the actual agro-infiltration study might then be used directly as a parent for breeding but the progenies obtained will not be transgenic as no genes are inserted into the genome. Alternatively, other plants which are genetically identical may be used as parents.

Progeny plants obtained after a floral dip treatment that have inserted the DNA fragment in the genome do not differ from GM plants obtained by other transformation methods.

ANNEX 16: TASK FORCE ON DETECTING AND IDENTIFYING CROPS PRODUCED WITH THE NEW PLANT-BREEDING TECHNIQUES - REPORT

NEW PLANT BREEDING TECHNIQUES CHALLENGES FOR DETECTION AND IDENTIFICATION

REPORT FROM THE "NEW TECHNIQUES TASK FORCE" (NTTF)

(FULL Final Version 15 December 2010)

The views expressed in this report are those of an expert task force and do not necessarily represent those of the European Commission or the Competent Authorities.

Introduction

Background

At the request of the Competent Authorities under Directive 2001/18/EC, a working group of Member States experts, the so-called "New Techniques Working Group" (NTWG) was established to analyse a non-exhaustive list of techniques for which it is unclear whether they would result in a genetically modified organism.

In its discussions, the NTWG noted that there is a growing interest in using biotechnology in such a way that the resulting plant or organism does not contain any genetic material from an organism that it could not breed with naturally or indeed, contain any new genetic material at all. Furthermore, in some cases the resulting changes are similar to those achievable with conventional breeding techniques and such organisms may be indistinguishable from their conventional counterparts. In particular, the following issue was foreseen: enforcement becomes more difficult if the resulting organisms are indistinguishable from their conventional counterparts or natural variants and cannot be detected to be the result of a genetic modification technique.

Establishment of the "New Techniques Task Force" - NTTF

Availability of validated detection methods is a regulatory requirement for the approval of GMOs under EU legislation. It was therefore decided that the possibilities for detecting crops produced with new plant breeding techniques should be investigated. The findings are described as part of this report.

In the EU, extensive experience on detection of genetic modification has been collected since the late 1990s, in particular on the basis of the regulatory requirements of the EU legislation on GMOs. Submission and validation of GMO detection methods are today an integral part of the EU regulatory approval process for GMOs since Regulation (EC) No 1829/2003 on GM food and feed provides that the application for authorisation should include, amongst others "methods for detection, sampling and identification of the transformation event".

Regulation (EC) No 1829/2003 also provides in particular that:

1. The European Union Reference Laboratory for GM Food and Feed (EU-RL - GMFF) referred to in Article 32 is the Commission's Joint Research Centre.

2. For its duties and tasks, the European Union Reference Laboratory (EU-RL) shall be assisted by the national reference laboratories referred to in Article 32, which shall consequently be considered as members of the consortium referred to as the "European Network of GMO laboratories" (ENGL).

MS	ORGANISATION	NTTF CONTACT	
BE	Scientific Institute of Public Health (IPH)	Sylvia Katia	Broeders Pauwels
BE	Institute for Agricultural and Fisheries Research (ILVO)	Marc	De Loose
CZ	Crop Research Institute (VURV)	Jaroslava	Ovesna
DE	Federal Office of Consumer Protection and Food Safety (BVL)	Hans-Jörg	Buhk
NL	Institute of Food Safety (RIKILT)	Theo W.	Prins
PL	Plant Breeding and Acclimatisation Institute (IHAR)	Slawomir	Sowa
SI	National Institute of Biology (NIB)	Mojca	Milavec
UK	Food and Environment Research Agency (FERA)	Christine	Henry
EU	Joint Research Centre (JRC) Institute for Health and Consumer Protection (IHCP)	Damien Marc	Plan Van den Bulcke

Note: other European Commission services who are also working on new plant breeding techniques (like the JRC Institute for Prospective Technological Studies (IPTS) and DG SANCO, the Directorate-General for Health and Consumers) have been associated and regularly informed about the activities of the NTTF.

For this investigation on detection and new plant breeding techniques we established a "New Techniques Task Force" (NTTF). In order to benefit from the expertise already existing on GMO detection and analysis within the European Network of GMO Laboratories (ENGL)[58], eight technical experts were selected amongst the ENGL members to join the NTTF (see table above).

Methodology followed by the NTTF

Between April and November 2010, the NTTF held 11 conference calls and 3 meetings (including a meeting with industry representatives in November 2010). In December 2010, the present technical report on "New Plant Breeding Techniques and Challenges for Detection and Identification" was produced.

For this evaluation the NTTF agreed in particular to:

- focus on technical issues related to detection and identification of genetic modifications resulting from new plant breeding techniques (i.e. not to include discussions on future regulatory decisions on new plant breeding techniques).

- focus on the list of new plant breeding techniques addressed in the NTWG, with the exception of synthetic genomics which is not yet relevant for plant breeding, and therefore to focus on the following seven techniques:
  1. Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
  2. Oligonucleotide directed mutagenesis (ODM)
  3. Cisgenesis and intragenesis
  4. RNA-dependent DNA methylation (RdDM)
  5. Grafting (on GM rootstock)
  6. Reverse breeding
  7. Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

- focus not only on the detection of a genetic modification but more importantly on the identification of the genetic modification as intentionally introduced by a new technique.

58 The ENGL is a consortium of national reference laboratories (including around 100 members) which was established by Regulation (EC) No 1829/2003 on GM food and feed and which is assisting the European Union Reference Laboratory for GM food and feed (EU-RL GM FF) in its duties, in particular validation of GMO detection methods.

Enforcement becomes more difficult if the resulting organisms are indistinguishable from their conventional counterparts or natural variants and cannot be detected to be the result of a genetic modification technique. Therefore, the NTTF decided to make an important distinction between the concepts of "detection" and "identification" which should be understood, for the purposes of this NTTF report, as follows:

DETECTION: detection of a genetic modification means that it is possible to determine the existence of a change in the genetic material of an organism (for instance at the level of DNA through the presence of a novel DNA sequence) by reference to an appropriate comparator.

IDENTIFICATION: identification of a genetic modification means that it is possible not only to detect the existence of a change in the genetic material of an organism (see detection text before) but it is also possible to identify the genetic modification as intentionally introduced by a new technique.

For each individual new technique, the NTTF also agreed to consider the following two scenarios:

WITH PRIOR KNOWLEDGE: refers to cases where information is available (for instance at the level of DNA sequence) on the product resulting from the use of a new plant breeding technique. This information may be made available for instance from the company having developed the product.

WITHOUT PRIOR KNOWLEDGE: refers to cases where no information at all is available on the product resulting from the use of a new plant breeding technique. This situation may be compared with the challenges already raised today for the detection of "unknown" GMOs.

Note: a new document from the ENGL on "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials" is under preparation and is expected to be published in 2011. It will address in detail the challenges raised by the detection of GMOs unauthorised in the EU and will propose in particular a GMO classification based on the level of available knowledge concerning the genetic structure, from "GMOs fully characterised" (knowledge level 1) to "GMOs transformed with only novel genetic elements" (knowledge level 4). For this latter category "GMOs transformed with only novel genetic elements", it is anticipated that the "use of only novel elements will make the GMO undetectable with any of the currently used detection methods and will imply that the GMO is "unknown" for the analyst". This upcoming ENGL publication will therefore provide further detailed information on the challenges raised by the detection of "unknown" GMOs, which may be relevant to the ones raised in the present report under the scenario "without prior knowledge".

- focus on the analysis of crops developed (i.e. not taking into account processed products and mixtures thereof).

The NTTF recognised that the type of material (matrix) to be analysed will have an influence on the analytical capacity of any detection approach used and that different detection possibilities and situations will arise along the complete supply chain (from seeds to grains, food/feed processing and final processed food/feed products).

The influence of the type of material (matrix) to be analysed on the analytical capacity has been addressed, amongst others, in various guidance documents developed by the EU-RL GMFF and the ENGL. For instance the document on "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" includes in the method acceptance criteria the topic "Applicability" i.e. "the description of analytes, matrices and concentrations to which the method is applied". The method description should include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations. This topic is also addressed in specific EU legislative texts related to GMO method validation and information about the method, like Annex I of regulation (EC) No 641/2004.

The NTTF recognised as well that sensitivity of a particular detection method will also be negatively influenced when a mixture of plants (or even more a mixture of processed foods) has to be analysed in comparison to individual plants.

Further to these considerations and taking into account the mandate and timelines for developing its report on "New Plant Breeding Techniques and Challenges for Detection and Identification", the NTTF decided to focus the scope of its work and the contents of the present report at the level of individual plant material (i.e. without focusing on cases of processed products and mixtures).

Structure of the NTTF report

The main objective of the NTTF was to produce a technical report on the detection and identification challenges raised by the following seven techniques:

1. Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
2. Oligonucleotide directed mutagenesis (ODM)
3. Cisgenesis and intragenesis
4. RNA-dependent DNA methylation (RdDM)
5. Grafting (on GM rootstock)
6. Reverse breeding
7. Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

For consistency reasons, the NTTF agreed to use definitions of the above new plant breeding techniques which are in line with the ones used in the NTWG.

The NTTF also agreed that the contents of its technical report should be structured around two main sections addressing on one side "state-of-the art" for detection and identification of genetic modifications in plants and on the other side "specific considerations" for detection and identification of intentional genetic modifications by new plant breeding techniques. These two main sections correspond to the following Part 1 and Part 2.

Part 1: State-of-the art for detection and identification of genetic modifications in plants

Information concerning the genotype of plants can be obtained at different levels, e.g. at the level of DNA, proteins and metabolites. Modern analytical methods exist on all of these levels and the NTTF discussed their applicability for the detection and identification of crops developed through new plant breeding techniques.

This "State-of-the art" section considers therefore three general approaches to detect and identify genetic modifications:

1. DNA-based analysis
2. Protein-based analysis
3. Metabolite-based analysis

This section 1 was developed using existing knowledge and information on techniques available for GMO detection, in particular it is based on the activities of the EU-RL GMFF and of the ENGL, as well as activities of standardisation bodies like ISO and CEN.

Part 2: Specific considerations for detection and identification of intentional genetic modifications by new plant breeding techniques

Based on section 1, the NTTF comes to the general conclusion that DNA amplification-based methods (PCR) are the most appropriate for detection and identification of genetic modifications.

The EU regulatory approach based on validation of GMO event-specific PCR methods can be considered as the "reference" or "baseline" for detection and identification of products obtained through a deliberate genetic modification technique, be it through genetic engineering (like GMOs defined under Article 2 (2) in conjunction with Annex IA Part 1 of Directive 2001/18/EC) or through a new technique.

In this section 2 we report the possibilities of detection and identification for each of the seven individual new plant breeding techniques. Based on current available detection methods summarised before, the "reference" or "baseline" for this analysis was therefore the PCR-based approach for detection of GMOs (known or unknown).

For each specific new plant breeding technique the following information is given:

1. Definition of the individual New Technique
(including if needed some general considerations)

2. Detection and identification with prior knowledge

This scenario refers to cases where information is available (in particular at the level of DNA sequence) on the product resulting from the use of a new plant breeding technique. This information may be made available for instance from the company having developed the new product (plant). Cross-reference is made to Chapter 7.1 which includes details on the type of information required to allow detection and identification of genetic modification.

3. Detection and identification without prior knowledge

This scenario refers to cases where no information at all is available on the product resulting from the use of a new technique. It is to be noted that in the case of "unknown" GMOs (i.e. GMOs for which no information is available for instance because no regulatory application has been filed) detection and identification are challenging[59].

4. Conclusions

The conclusions summarise the opinion of the NTTF regarding the possibility to detect and more importantly to identify products from the various individual new plant breeding techniques i.e. the possibility to differentiate them from products resulting from natural mutations or obtained from other breeding techniques, e. g. mutagenesis.

Work Plan of the NTTF

The NTTF worked according to the following timelines, mainly through conference calls with some face-to-face meetings held when needed:

12 April 2010:	NTTF conference call No1
3 May 2010:	NTTF conference call No2
17 May 2010:	NTTF meeting No1 hosted by JRC IHCP in Ispra, Italy
27-28 May 2010:	NTTF participation to the workshop on New Plant Breeding Techniques organised by JRC IPTS in Sevilla, Spain
14 June 2010:	NTTF conference call No3
29 June 2010:	NTTF conference call No4
27 July 2010:	NTTF conference call No5
17 August 2010:	NTTF conference call No6
August 2010:	NTTF interim report
8 September 2010:	NTTF meeting No2 hosted by JRC IHCP in Ispra, Italy

59 A new document from the ENGL on "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials" is under preparation and is expected to be published in 2011. This upcoming ENGL publication will provide further detailed information on the challenges raised by the detection of "unknown" GMOs, which may be relevant to the ones raised in the present report under the scenario "Without prior knowledge".

5 October 2010: NTTF conference call No7
19 October 2010: NTTF conference call No8
26 October 2010: NTTF conference call No9
29 October 2010: NTTF conference call No10
10 November 2010: NTTF meeting No3 hosted by JRC IHCP in Ispra, Italy (including representatives from industry)
26 November 2010: NTTF conference call No11
December 2010: NTTF final report

Part 1: State-of-the art for detection and identification of genetic modifications in plants

1 Introduction

The genetic information of all organisms (including viruses) is stored in its nucleic acid (usually double stranded Deoxyribonucleic acid (DNA), or Ribonucleic acid (RNA) in the case of some viruses) in a code of a specific sequence of four different nucleotides. This information gets turned into a functional trait by two consecutive biological processes.

In the first step of "transcription", RNA is formed. This single stranded molecule is a complementary copy of the DNA sequence with the difference that, wherever DNA contains the nucleobase thymine in its sequence, RNA contains the nucleobase uracil instead. Three different major forms of RNA are synthesised: messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

All three types of RNA are required for the second step, protein synthesis, which is the "translation" of the genetic information into a sequence of amino acids, a polypeptide or protein. The mRNAs are used as templates for protein synthesis and determine the amino acid sequence of proteins. The tRNAs and rRNAs are molecules needed to constitute a functional protein synthesis machinery.

The synthesised proteins serve different functions of the cell, as structural elements, transporters, regulators and enzymes. Especially the latter two are involved in the synthesis of other structural components of the cell, the lipids and the polysaccharides.

With regard to genetic modification - be it by natural mutation or by genetic engineering - information concerning the genotype of the organism can be obtained at each level of the process of conversion of genetic information into structural and functional trait: be it at the level of DNA, the level of RNA, the level of proteins, the level of cellular non-nucleic acid or non-protein substances and finally at the level of phenotypes.

However, the conclusions that can be drawn from the detection of a genetic modification at these different levels above may vary considerably. The following example will illustrate this.

Soybean plants, which normally are sensitive to a certain herbicide, exhibit resistance against this herbicide. Different explanations are possible. The plants may have, through genetic engineering, obtained a gene encoding a herbicide-degrading enzyme; alternatively, the plants may have undergone spontaneous natural mutations which either prevent uptake of the herbicide into the plant or alter the target of the herbicide within the plant cells. Different analytical options are possible to exclude spontaneous mutations and to confirm the genetic modification as introduced by genetic engineering: at the level of the gene encoding the enzyme, of the mRNA transcribed from the gene or of the protein expressed.

The meaningfulness of assays also needs to be considered when designing assays to detect modifications introduced by genetic engineering. In the example above, determination of the phenotype is of no value. In this case, the various possible assays should be based on the analysis of DNA, mRNA or enzymes.

Another fact that must be considered is the degeneration suffered by the genetic information during conversion into structural and functional traits.

The only biological process resulting in an exact 1:1 copy of the DNA is replication. Transcription usually yields 1:1 copies of the transcribed DNA regions. However, non-transcribed DNA regions will never show up at the RNA level. Furthermore, especially in higher organisms, the primary transcript produced by the step of transcription may be altered by an editing process in which specific sequences - called introns - are deleted from the primary RNA to form the actual mRNA.

During translation, further information gets lost or is obscured:

- Within the process of transcription, only part of the mRNA is translated into a protein (the regions translated are called open reading frames).

- A frame of three mRNA nucleotides (a codon) is required to encode one amino acid. Three nucleotides out of four offer the possibility to form 64 different combinations. However, as only 20 amino acids are used for protein synthesis, several codons code for the same amino acid. Actually, each of the three amino acids serine, leucine and arginine is encoded by six different codons. Only methionine and tryptophan are each encoded by just one codon. Thus, the amino acid sequence of a protein is only partly suitable for deducing the nucleotide sequence of the mRNA.

- Many proteins are subject to post-translational processing. One result of this processing may be the removal of part of the polypeptide chain. It is therefore obvious that no information on the mRNA or DNA sequence of the removed polypeptide parts can be deduced from the mature protein.

Sequence analyses of RNA and protein may therefore allow drawing only some partial conclusions on the DNA sequence. As shown above, such analyses may indicate the presence of a genetic modification. However, no definitive information on the true nature of the modification can be obtained, in particular because of the loss of information during the conversion from DNA to RNA and to proteins. On the other hand, analyses of other constituents of the cell (lipids, carbohydrates, metabolites and solutes) and of the phenotypes do not provide at all any information on the DNA sequence.

Thus, it can be concluded that DNA is the ideal target molecule for detecting and identifying unambiguously a change as the result of the use of a genetic modification technique.

Furthermore, it should be noted that the introduction of a foreign gene into the DNA of an organism can be unambiguously detected only at the level of DNA. For instance the presence of a bacterial enzyme within an extract of a plant may be the result of a contamination. As long as the enzyme has not been altered by a post-translational process specific for bacteria, the protein itself will not reveal whether it was expressed in a plant or in a bacterium. However, the corresponding gene, cloned in a vector construct, transformed into the plant, and integrated into the plant DNA, can always be identified as a foreign gene, because it is flanked by DNA sequences which do not naturally flank this gene. An assay targeting the fusion sites of two DNA sequences of different origin, therefore, unambiguously identifies a product of a genetic engineering process: unique DNA sequences which are exclusively present in the specific recombinant DNA construct and nowhere else.

Some genetic modification techniques may involve the deliberate replacement of just one nucleotide for another. DNA-based methods are capable of detecting such minor alterations but require information on the nucleotide sequence in the direct vicinity of the modification. However, even if detectable, such minor modifications are difficult to differentiate from naturally occurring mutations. Changes at single nucleotide level are therefore always difficult to identify as being the result of a genetic modification technique. To date several different methods have been developed for an efficient genotyping for the detection of allelic genes. They can in principle be employed to detect natural occurring or induced changes of one or a few nucleotides. Essentially the current methods can be grouped according to their basic principles: allele-specific oligonucleotide ligation; allele-specific primer extension; allele-specific hybridisation; and

allele-specific cleavage reactions. Some of the methods can be combined with different methods of signal detection and signal amplification (e.g. mini-sequencing, chip-based method, fluorescence resonance energy transfer label). Any of these methods requires however some prior knowledge on the target DNA sequence.

In a genome of a size of *Escherichia coli* K12 i.e. 4.64×10^6 base pairs (bp), any 10 bp oligonucleotides (1.05×10^6 different sequences possible) should appear with a likelihood of roughly $4.64 \times 10^6 : 1.05 \times 10^6 = 4.42$, under the assumption that the nucleotides in the genome are dispersed randomly (and even though the nucleotides may not be actually dispersed purely randomly such calculation provides a helpful estimation). Therefore, a target sequence for the *E. coli* genome should go beyond 10 nucleotides and be approximately 15 nucleotides long to be statistically considered as unique.

Based on the same kind of assumption, a target sequence for a plant genome of the size of *Zea mays* for instance (2.5×10^9 bp / haploid genome) would require a size of approximately 20 nucleotides to be statistically considered as unique and therefore to be identified as the result of a genetic modification technique.

It can therefore be assumed that in the case of a plant genome, information on DNA sequence of at least 20 nucleotides is needed to be in a position to consider a certain DNA sequence as unique and to identify it as the result of a deliberate genetic modification technique.

It is self evident that any minor modification either deliberately introduced or occurring naturally cannot be easily detected without prior knowledge i.e. if no information at all on the particular DNA sequence is available. Without prior knowledge, only if a considerable large piece of foreign DNA is introduced, such modification can be detected and identified as the result of a deliberate genetic modification technique because of its unique nature.

Note: to be expressed in an organism, any novel sequence is to be fused to appropriate transcription signals that are functional in that organism. As to date, the number of suitable transcription elements is limited, the corresponding sequences can be used for the screening of the presence of novel modifications. In this respect, combining multiple elements in a screening approach can provide detailed information on the set of modified organisms present in a sample. The interpretation of the results obtained by such an approach is to be supported by an a priori defined reference table listing the occurrence of the screening targets in already characterised modified organisms and by comparing the screening results with the outcomes as expected from the reference table.

As will be further detailed in the following chapters, any DNA-based detection method relies on the availability of at least a minimum of information about the target DNA sequence. Therefore, even considering all existing sophisticated DNA-based analytical methods, one must conclude that no reliable method is available to identify an unknown modification.

2 DNA-based analysis

DNA-based analysis targets the novel DNA sequences introduced into the crop. These methods show the absence or presence of novel plant material in a sample and some of them can also measure the relative quantity (percentage) in a tested sample.

2.1 DNA amplification-based methods (PCR)

Amplification techniques involve denaturation of the double stranded nucleic acid followed by annealing of a short oligonucleotide (primer) and primer extension by a DNA polymerase. The most common technique is the polymerase chain reaction (PCR) technique, employing a thermo-stable DNA polymerase.

PCR is the most commonly used technique for GMO detection. Figure 19 details the different levels of specificity of GMO detection possible with PCR technology (from screening to construct-specific and event-specific), depending on the type of DNA sequence information available.

Nucleotide sequence specific oligonucleotides, binding to the target DNA to the left and to the right of the target site, allow an enzyme to prolong the oligonucleotide primers and thereby to amplify specifically the DNA fragment between the primers. Repeated cycles of the reaction lead to a logarithmic amplification of the fragment. The design of specific primers depends on knowledge of the precise and comprehensive DNA sequence information of the actually integrated DNA.

If the method is to specifically detect and identify a certain transformation event (event-specific method), information about the inserted DNA sequence and about the 3' and 5' flanking plant genome sequences is required (Fig. 2).

For element-specific, PCR-based screening, and construct-specific detection, the DNA sequences of the inserted elements and gene constructs are targeted, respectively.

PCR-based detection and particularly the quantitative measurement of the GM content in a sample actually involves the use of two PCR systems, one for determination of the inserted GM-derived DNA sequence and another system specific for an endogenous, plant-taxon specific reference gene sequence (Fig. 20). The latter also serves as a control for the quality and quantity of the extracted DNA.

2.1.1 Conventional qualitative PCR

Conventional PCR methods are mainly used for qualitative testing to obtain yes/no answers concerning the presence of GM plant material. PCR products are analysed by agarose or polyacrylamide gel electrophoresis and visualised using UV fluorescence with ethidium bromide as fluorophore or by other means.

It may be necessary to confirm GM-positive test results by further analyses, either by restriction analyses, Southern hybridisation or DNA sequencing.

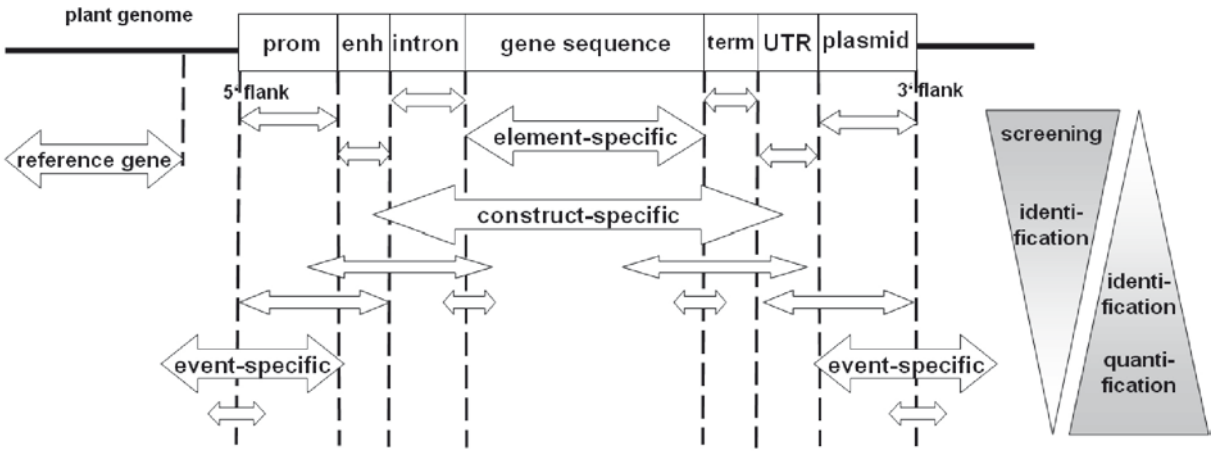
The important performance criteria for qualitative PCR methods are the sensitivity in detecting the DNA sequences and the specificity for the targeted DNA segment. At optimal reaction conditions a limit of detection (LOD) of 1 - 10 copies of the target sequence can be achieved in less than 40 PCR cycles. Practically the LOD of the PCR method should allow that the presence of the target sequence is detected in at least 95% of the time, with less than 5% false negative results. The length of the amplified product influences the PCR performance and should therefore be selected in a way that it matches to the size range of DNA fragments which can be extracted from the sample matrix. For raw materials like seeds or leaves containing less fragmented DNA a broader range of PCR product size up to maximally 250 bp is applicable, whereas for processed food or feed with higher DNA fragmentation the PCR product should be ideally 80 - 150 bp. The specificity of the method should be tested theoretically by sequence similarity search with the primer sequences against nucleic acid sequence databases and empirically by testing the target event(s), very similar non-target events and different non-modified plants in order to confirm that the primers can discriminate between the target and closely related non-target sequences. For reference gene-specific PCR methods, different varieties should be tested to demonstrate that the target sequence is conserved between different plant lines.

2.1.2 Quantitative Real-Time PCR

The most preferred technique to quantify GM material in a sample is Real-Time PCR. It allows the detection and measurement of increasing fluorescence proportional to the amount of amplification products generated during the PCR process. Of the various chemistries TaqMan fluorogenic probes are most commonly applied in Real-Time PCR-based detection and quantification of GM plant materials. Real-Time PCR is mainly used for quantification purposes, but it is increasingly utilised also for qualitative testing to screen or to identify the GM event.

The limit of quantification (LOQ) of a Real-Time PCR method depends on the optimisation of the PCR detection method and on the accepted standard deviation of the measurement. The LOQ is experimentally determined during method validation and should reach 30 - 50 target molecules, which is close to the theoretical prediction. The LOD / LOQ values depend primarily on the characteristic plant genome size (C value).

Figure 19: Schema of a transformation construct comprising seven elements inserted into a plant genome through a certain transformation event and, therefore, flanked by specific DNA sequences of the plant genome.



Arrows of the upper four rows indicate regions suitable for element-specific detection. Such screening assays target widely used genetic elements like promoters.
Arrows in the following three rows in the middle indicate regions suitable for construct-specific detection. Construct-specific assays are designed to comprise a junction between different elements of the inserted sequence.
Arrows in the two rows at the bottom indicate regions suitable for event-specific detection. Event-specific assays are the most specific ones and are constructed over a junction between the host and the inserted sequences with specific primers for the inserted gene and the flanking genomic sequence.
An example for a reference gene is indicated. The two triangles at the right hand side indicate a gradient of suitability for screening, identification, and quantification.

Note: the EU-RL GMFF and the ENGL have developed various guidance documents on PCR methods, including in particular the document on "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (available at http://gmo-crl.jrc.ec.europa.eu/default.htm) which defines the acceptance criteria to be met before a method can enter the EU validation process. Parameters addressed in this guidance document include Applicability, Practicability, Specificity, Dynamic Range, Trueness, Amplification Efficiency, Precision, LOD, LOQ and Robustness.

Figure 19 details the different levels of specificity of GMO detection possible with PCR technology (from screening to construct-specific and event-specific), depending on the type of DNA sequence information available.

2.1.3 Conclusions for detection by PCR-based methods

Any PCR-based method relies on the availability of a certain minimum of information about the target DNA sequence. Some information needs to be known about the inserted DNA sequence and about the 5' and/or 3' neighbouring genomic DNA sequence in order to allow the identification of an intentional genetic modification (see further details below).

Without prior knowledge, reliable identification of a genetic modification is not possible even with the most sophisticated available methods for DNA analysis.

PCR-based analytical methods for the detection of intentionally modified DNA sequences provide high sensitivity and specificity. PCR supports the development of specific methods that allow the detection as well as the identification of intentionally modified DNA, i.e. plants with known intentional modifications can be differentiated for instance from plants presenting similar phenotypes and from plants possibly presenting a similar DNA modification through natural mutation.

2.1.3.1 Insertions larger than 80 bp

For the detection and the identification of an insert, the primers and probe need to be designed within the insert. Large inserts can be detected and identified when at least 80 bp of the inserted sequence is known.

For event-specific identification, a sufficient part of the sequence of the insert as well as a part of the adjacent sequence must also be known, in order to be able to design an event-specific primer pair and a probe. This information is a prerequisite for an unambiguous identification of an intentional genetic modification.

2.1.3.2 Short insertions

PCR-based methods are also capable to detect and identify short insertions of less than 80 bp. In this case specific primers are designed in order to bind to sequences including the insert and its flanking regions sites or to bind only to sequences directly flanking the insert. Irrespective of the number of modified base pairs, the specific primers should be at least approximately 20 nucleotides long and specific in sequence for the modification and its direct vicinity. In order to identify a short intentional modification and to differentiate it from a possible natural mutation, information on the modified sequence and the nucleotide sequence in its direct vicinity is required for the design of specific primers.

2.1.3.3 Modification of one or a few nucleotides

Intentional modifications of a single or a few nucleotides can in principle be detected. Information on the site of the modification and the nucleotide sequence in its direct vicinity of approximately 20 bp (including the site of modification) is necessary to ensure in principle the uniqueness of the sequence forming the newly created junction in the genome. For the amplification of this unique sequence by PCR further information upstream and downstream is required for the design of primers. If this 20 bp string matches with a repetitive sequence in the genome it cannot however unambiguously characterise the location of the modification.

2.1.3.4 Deletions

Deliberate modifications by deletions can also be detected in a similar way as described for modifications by short insertions. Information on the site of the deletion and the nucleotide sequence in its direct vicinity of approximately 20 bp including the site of deletion is necessary to ensure in principle the uniqueness of the sequence forming the newly created junction in the genome. For the amplification of this unique sequence the same requirement applies as for modification of a single or a few nucleotides. If this 20 bp

string matches with a repetitive sequence in the genome it cannot however unambiguously characterise the location of the modification.

2.2 DNA Sequencing

DNA sequencing allows determining the order of the nucleotide bases adenine, guanine, cytosine, and thymine in a DNA strand.

DNA sequencing is most commonly done on PCR amplified or cloned DNA fragments.

Determining the DNA sequence is useful in basic research studying fundamental biological processes, as well as in applied fields such as diagnostic and detection or forensic research.

2.2.1 Chemical sequencing (Maxam-Gilbert)

In 1976-1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. Also sometimes known as 'chemical sequencing', this method originated in the study of DNA-protein interactions (foot printing), of nucleic acid structure and of epigenetic modifications to DNA. Maxam-Gilbert sequencing rapidly became more popular, as purified DNA could be used directly. However, with the development and improvement of the chain-termination method (see below), Maxam-Gilbert sequencing has fallen out of favour due to its technical complexity, extensive use of hazardous chemicals, and difficulties with scale-up. In addition, unlike the chain-termination method, chemicals used in the Maxam-Gilbert method cannot easily be customised for use in a standard molecular biology kit.

2.2.2 Chain-termination methods

While the chemical sequencing method of Maxam and Gilbert was orders of magnitude faster than previous methods, the chain-terminator method developed by Sanger was even more efficient, and rapidly became the method of choice. The Maxam-Gilbert technique requires the use of highly toxic chemicals and large amounts of radiolabel DNA, whereas the chain-terminator method uses fewer toxic chemicals and lower amounts of radioactivity. The key principle of the Sanger method was the use of dideoxynucleotides triphosphates (ddNTPs) as DNA chain terminators.

The chain-termination methods have greatly simplified the amount of work and planning needed for DNA sequencing. However some sequencing problems can occur with them, such as non-specific binding of the primer to the DNA, affecting accurate read out of the DNA sequence. In addition, secondary structures within the DNA template, or contaminating RNA randomly priming at the DNA template can also affect the fidelity of the obtained sequence.

2.2.2.1 Dye-terminator sequencing

Labelling of the chain terminators with a different dye is used in a method commonly called 'dye-terminator sequencing'. The major advantage of this method is that the sequencing can be performed in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with a different fluorescent dye, each fluorescing at a different wavelength. This method is attractive because of its greater expediency and speed and is now the mainstay in automated sequencing with computer-controlled sequence analyzers (see below). Its potential limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. This problem has largely been overcome with the introduction of new DNA polymerase enzyme systems and dyes that minimise incorporation variability,

as well as methods for eliminating "dye blobs", caused by certain chemical characteristics of the dyes that can result in artefacts in DNA sequence traces.

The dye-terminator sequencing method, along with automated high-throughput DNA sequence analysers, is now being used for the vast majority of sequencing projects, as it is both easier to perform and lower in cost than most previous sequencing methods.

2.2.2.2 Automation and sample preparation

Modern automated DNA sequencing instruments (DNA sequencers) can sequence up to 384 fluorescently labelled samples in a single batch (run) and perform as many as 24 runs a day. However, automated DNA sequencers carry out only DNA size separation by capillary electrophoresis, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms. Sequencing reactions by thermo cycling, cleanup and re-suspension in a buffer solution before loading onto the sequencer are performed separately and thus more laborious.

2.2.2.3 Large-scale sequencing strategies

Current methods can directly sequence only relatively short (300 - 1 000 nucleotides long) DNA fragments in a single reaction. The main obstacle to sequence DNA fragments above this size limit is insufficient power of separation for resolving large DNA fragments that differ only by one nucleotide in length.

2.2.2.4 High-throughput sequencing

The high demand for low cost sequencing has given rise to a number of high-throughput sequencing technologies. These efforts have been funded by public and private institutions as well as privately researched and commercialized by biotechnology companies. High-throughput sequencing technologies are intended to lower the cost of sequencing DNA libraries beyond what is possible with the current dye-terminator method based on DNA separation by capillary electrophoresis. Many of the new high-throughput methods use methods that parallelize the sequencing process, producing thousands or millions of sequences at once.

In vitro clonal amplification

As molecular detection methods are often not sensitive enough for single molecule sequencing, most approaches use an *in vitro* cloning step to generate many copies of each individual molecule. Emulsion PCR is one method, isolating individual DNA molecules along with primer-coated beads in aqueous bubbles within an oil phase. A PCR then coats each bead with clonal copies of the isolated library molecule and these beads are subsequently immobilized for later sequencing. Another method for *in vitro* clonal amplification is "bridge PCR", where fragments are amplified upon primers attached to a solid surface.

Parallelized sequencing

Once clonal DNA sequences are physically localized to separate positions on a surface, various sequencing approaches may be used to determine the DNA sequences of all locations, in parallel. "Sequencing by synthesis", like the popular dye-termination electrophoretic sequencing, uses the process of DNA synthesis by DNA polymerase to identify the bases present in the complementary DNA molecule. Reversible terminator methods use reversible versions of dye-terminators, adding one nucleotide at a time, detecting fluorescence corresponding to that position, then removing the blocking group to allow the polymerization of another nucleotide. Pyrosequencing also uses DNA polymerization to add nucleotides, adding one type of nucleotide at a time, then detecting and quantifying the number of nucleotides added to a given location through the light emitted by the release of attached pyrophosphates.

"Sequencing by ligation" is another enzymatic method of sequencing, using a DNA ligase enzyme rather than polymerase to identify the target sequence. This method uses a pool of random oligonucleotides labelled according to the sequenced position. Oligonucleotides are annealed and ligated. The preferential ligation by DNA ligase for matching sequences results in a signal corresponding to the complementary sequence at that position.

2.2.3 Other sequencing technologies

Other methods of DNA sequencing may have advantages in terms of efficiency or accuracy. Like traditional dye-terminator sequencing, they are limited to sequencing single isolated DNA fragments.

"Sequencing by hybridisation" is a non-enzymatic method that uses a DNA microarray. In this method, a single pool of unknown DNA is fluorescently labelled and hybridized to an array of known sequences. If the unknown DNA hybridizes strongly to a given spot on the array, causing it to "light up" then that sequence is inferred to exist within the unknown DNA being sequenced.

Mass spectrometry can also be used to sequence DNA molecules. Conventional chain-termination reactions produce DNA molecules of different lengths and the length of these fragments is then determined by the mass differences between them (rather than using gel separation).

Resequencing or targeted sequencing is utilised for determining a change in DNA sequence from a "reference" sequence. It is often performed using PCR to amplify the region of interest (pre-existing DNA sequence is required to design the PCR primers). Resequencing uses three steps: extraction of DNA or RNA from biological tissue, amplification of the RNA or DNA (often by PCR), followed by sequencing. The resultant sequence is compared to a reference or a normal sample to detect mutations.

2.2.4 Conclusions for detection by DNA sequencing

The detection of intentional modifications by DNA sequencing also requires prior knowledge of the nucleotide sequence of the introduced modification and its vicinity, as described for DNA amplification-based methods (most of the DNA sequencing techniques also include a PCR DNA-amplification step).

Developments in the field of DNA sequencing are rapidly expanding. However it can be concluded that today whole genome sequencing is not applicable for routine analyses of genetic modifications (in particular analysis of the huge amount of data generated is still challenging and costs are also still quite high).

2.3 DNA hybridisation-based methods

The development of DNA:DNA hybridisation on a solid support was an important development for the characterisation of nucleic acids.

Hybridisation-based methods rely on the fact that a DNA double helix molecule will become single stranded at elevated temperature. At a temperature below its "melting point" the two complimentary nucleotide sequence strands will fuse (hybridise) to each other as soon as they meet at complimentary stretches of sequence.

2.3.1 Southern blot

DNA:DNA hybridisation immobilised to a solid support is still an important technique for the characterisation of nucleic acids. This "Southern blot" procedure includes agarose gel electrophoresis for size separation of DNA fragments, followed by transfer and immobilisation of the separated DNA fragments onto a membrane

with subsequent hybridisation with a labelled DNA probe and detection through either radioactive labelling or e. g. chemiluminescence.

The generation of a specific signal based on DNA:DNA hybridisations is highly dependent on variable parameters such as transfer efficiency from the agarose gel to the membrane, degree of sequence homology, incubation time, buffer conditions, and temperature.

Southern blotting methods can support common DNA amplification methods (e.g. PCR) by verifying amplified DNA sequences through restriction enzyme digestion and subsequent hybridisation to target sequence-specific probes.

Although low sensitivity is the major restriction of this technique, it is still useful to elucidate the genomic areas of an inserted genetic modification or to verify the structure of the inserted DNA. However, due to its limitations this technique alone does not provide the necessary performance to detect low amount of genetically modified material.

2.3.2 Microarray

Microarray technology is based on hybridisation of complementary nucleotide strands (DNA or RNA). A large number of probes representing genes are placed on a very small surface. A micro array is normally between 1-4 cm^2 in size and contains between a couple of tens and several tens of thousands of gene representatives (low density array between ten and a couple of thousands, high density array between a thousand and several tens of thousands). The gene representing DNA oligonucleotides are immobilised onto a support such as glass, silicon or nylon membrane. Each spot on the chip is representative for a certain gene (or transcript). A specific hybridisation of the labelled sample DNA onto fixed capture nucleotides provides information about quality as well as quantity of potential genetic modifications, mostly analysed using fluorescence tags, permitting a profiling of different genetic modifications in one step.

Besides optical detection methods several other have been considered and applied. In particular, specially developed functional piezoelectric affinity sensors can detect DNA-hybridisation directly by oligonucleotides which are immobilised on electrode surfaces generating piezoelectric signals, and thus indicating the presence of modified DNA sequences. But in order to be sufficiently sensitive and to identify the modification by micro array technique the target DNA needs to be amplified preferably by PCR. Therefore the prerequisites for detection by PCR apply also for detection by microarrays.

2.3.3 Conclusions for detection by hybridisation-based methods

The detection of intentional modifications by hybridisation-based methods also requires prior knowledge of the nucleotide sequence of the introduced modification and its vicinity, as described for DNA amplification-based methods.

All in all, it can be concluded that DNA hybridisation methods are not practical for routine analyses of genetic modifications (in particular DNA hybridisation techniques offer low sensitivity compared to amplification-based methods).

3 Protein-based analysis

The genetic information in a plant (DNA) is translated into proteins via an intermediate (RNA). Proteins are made up of amino acids. Each amino acid is specified by a triplet code of the DNA and transcribed RNA. The sequence of amino acids specify the three dimensional structure of the protein and also its functionality, although some changes can occur after the production of the protein and are referred to as post-translational modification.

Proteins in plants can for example act as enzymes driving the metabolism of the cell: respiration, photosynthesis, gene replication, etc., or act as structural proteins.

3.1 Sequencing using Mass Spectrometry

In the world of protein Mass Spectrometry (MS), there is not one, all-purpose workflow (see following options). Some researchers separate proteins on two-dimensional gels (2-D), while others use Liquid Chromatography (LC). Some still identify proteins using peptide mass fingerprinting, while others sequence using tandem mass spectrometry.

Mass spectrometers for protein and peptide analysis can be configured for use with either electro spray ionisation (ESI) or matrix-assisted laser-desorption ionisation (MALDI) (Figure 20), both of which are "soft" techniques that enable the transfer of intact proteins and peptides into the gas phase without fragmentation. ESI spectra are considerably more complex than MALDI spectra, with a collection of peaks per species: one for each charged state. However, by producing multiply charged ions, ESI makes larger proteins accessible to analysis than does MALDI. In addition, multiply charged ions also are more amenable to tandem mass spectrometric analysis.

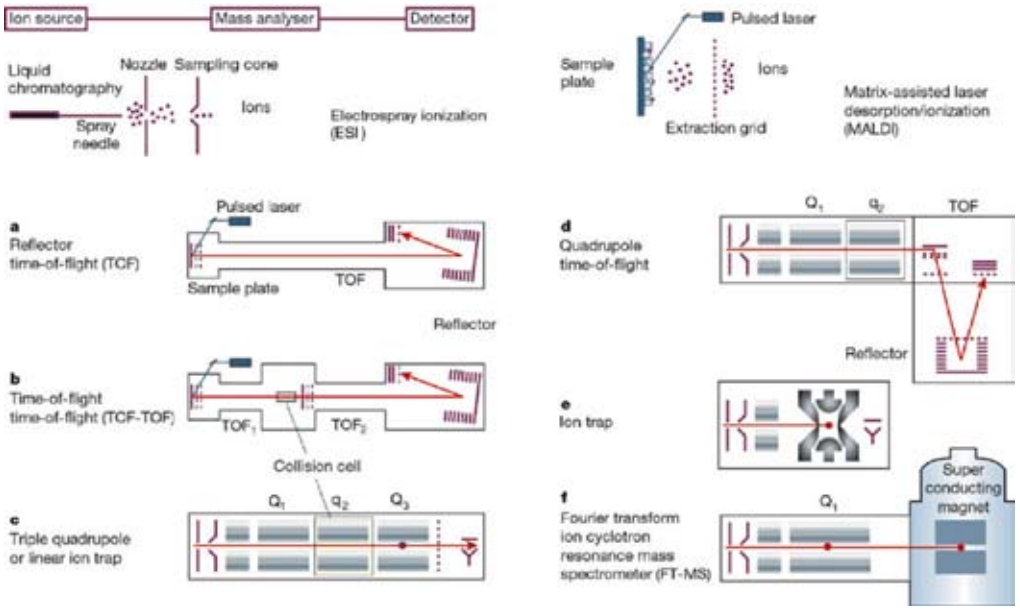
Two fundamental strategies for protein identification and characterization by mass spectrometry currently are employed in proteomics:

- In bottom-up approaches, purified proteins, or complex protein mixtures, are subjected to proteolytic cleavage, and the peptide products are analysed by MS.
- In top-down approaches, intact protein ions or large protein fragments are subjected to gas-phase fragmentation are analysed by MS.

The most straight forward use of mass spectrometry in proteomics would be to ionise a mixture of proteins, measure the masses of the ions formed, and use the mass-to-charge ratios to identify and quantify every protein. This approach, called "top-down" proteomics requires extremely high mass resolution and accuracy to deal with large proteins. However, measurement accuracy decreases as the absolute mass increases, making accurate identification of large proteins difficult. Many different proteins may have masses within the margin of error for these measurements. Post-transitional modifications make analysis more complicated since many post-transitional modifications change the mass of a protein but do not change its sequence.

An alternative approach is "bottom-up" or "shotgun" proteomics, which involves protease digestion to chop the proteins (usually previously separated by 2-D gel techniques) up into peptides (short sequences of amino acids) before identification. Bottom-up proteomics has three major advantages over the top-down approach. First, as mass spectrometers are more accurate for smaller masses, they are better at resolving small peptides rather than large proteins. Second, the bottom-up approach also greatly reduces the chance that post-translational modifications will trip up the identification process: if enough peptides are unmodified, the protein can be identified, regardless of how many modifications were made to the other peptides. Finally, in tandem mass spectrometry the bottom-up approach yields easier-to-analyse fragment spectra because peptides have fewer components to break apart than do intact proteins.

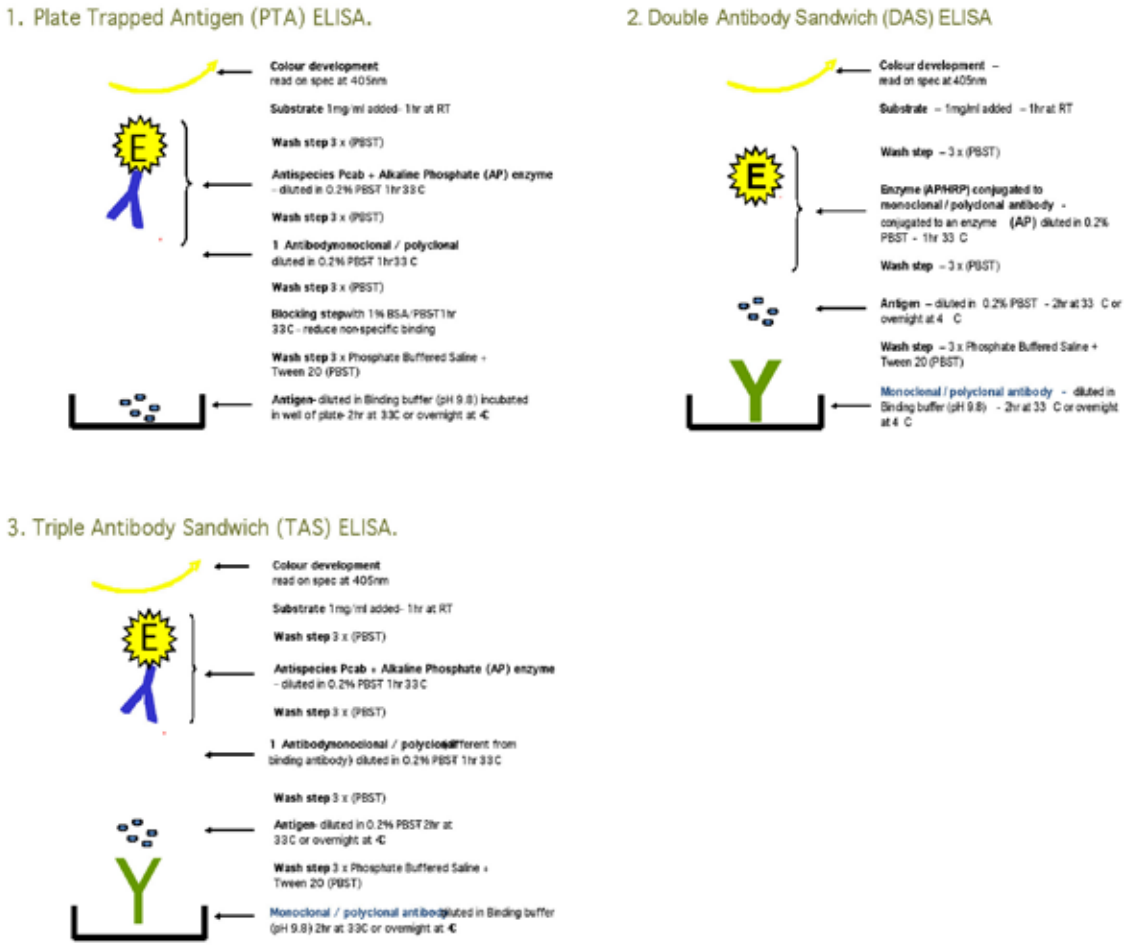
Figure 20: Mass spectrometers used in proteome research



The left and right upper panels depict the ionisation and sample introduction process in electro spray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The different instrumental configurations (a–f) are shown with their typical ion source. a, In reflector time-of-flight (TOF) instruments, the ions are accelerated to high kinetic energy and are separated along a flight tube as a result of their different velocities. The ions are turned around in a reflector, which compensates for slight differences in kinetic energy, and then impinge on a detector that amplifies and counts arriving ions. b, The TOF-TOF instrument incorporates a collision cell between two TOF sections. Ions of one mass-to-charge (m/z) ratio are selected in the first TOF section, fragmented in the collision cell, and the masses of the fragments are separated in the second TOF section. c, Quadrupole mass spectrometers select by time-varying electric fields between four rods, which permit a stable trajectory only for ions of a particular desired m/z. Again, ions of a particular m/z are selected in a first section (Q1), fragmented in a collision cell (q2), and the fragments separated in Q3. In the linear ion trap, ions are captured in a quadruple section, depicted by the red dot in Q3. They are then excited via resonant electric field and the fragments are scanned out, creating the tandem mass spectrum. d, The quadrupole TOF instrument combines the front part of a triple quadruple instrument with a reflector TOF section for measuring the mass of the ions. e, The (three-dimensional) ion trap captures the ions as in the case of the linear ion trap, fragments ions of a particular m/z, and then scans out the fragments to generate the tandem mass spectrum. f, The FT-MS instrument also traps the ions, but does so with the help of strong magnetic fields. The figure shows the combination of FT-MS with the linear ion trap for efficient isolation, fragmentation and fragment detection in the FT-MS section.

Note: trypsin, the protease most commonly used to digest protein samples into peptides, cleaves proteins at very predictable amino acid locations. Using software and databases, these masses are then compared to the theoretical masses of peptides coming from that organism, assuming the genome sequence is known. This process demands high sensitivity, mass resolution and accuracy.

Figure 21: Examples of typical ELISA systems



3.2 Immuno-based methods

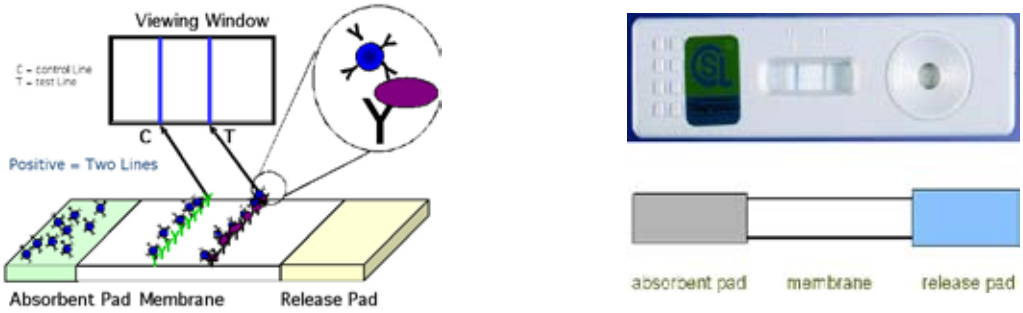
Many protein-based methods are often referred to as immunological techniques because the detection is often based on the immunological principle of conjugation between an antigen (the target) and an antibody (the probe specific to the antigen).

All of these methods rely on the use of antibodies for detection/identification of proteins. Therefore the target for production of antibodies must be immunogenic. This is not always the case. It may therefore be costly and time consuming to make antibodies. Most methods are difficult to make quantitative, although ELISA can be used in a quantitative mode provided pure standards are available. The use of monoclonal antibodies, as opposed to polyclonal antisera, gives greater specificity and more likelihood that small differences in proteins can be detected. Monoclonal antibodies are commonly developed using mice or rats, polyclonal antisera using rabbits.

3.2.1 Enzyme linked immunosorbent assays (ELISA)

Enzyme linked immunosorbent assays (ELISA) are very popular and efficient tools for rapid detection of a particular protein.

Figure 22: An example of a lateral flow kit format



In simple terms, in ELISA an extract containing the target protein is affixed to a surface (Plate Trapped Antigen (PTA) ELISA) either directly or using a trapping antibody (Double Antibody Sandwich (DAS) ELISA and Triple Antibody Sandwich (TAS) ELISA) and then a specific antibody is applied over the surface so that it can bind to the antigen - see Figure 21. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal, most commonly a colour change in a chemical substrate.

The specificity and sensitivity of the test depends on the type of antibodies used and on the testing system used. Monoclonal antibodies are generally more specific whereas polyclonal antibodies are less specific for the target protein concerned. The use of a TAS ELISA usually gives greater sensitivity than DAS ELISA or PTA ELISA because it includes an amplification step. The tests can be made quantitative provided standards exist. However relating protein quantity to a percentage of genetically modified organism for instance can prove difficult.

3.2.2. Lateral flow device (LFD)

Lateral flow devices (LFD) or lateral flow strips are related to ELISAs (see Figure 22). LFDs are again based on detection of the protein using antibodies, using similar principles to that of ELISA. An extraction of the GM plant for instance is placed at one end of a membrane and moved through this by diffusion using an absorbent pad. As the protein front reaches a line of specific antibody it reacts with this and the conjugate to produce a colour reaction. Newer types of LFD systems can be semi-quantitative. The main strength of the technique is as a screening technique for use in field conditions.

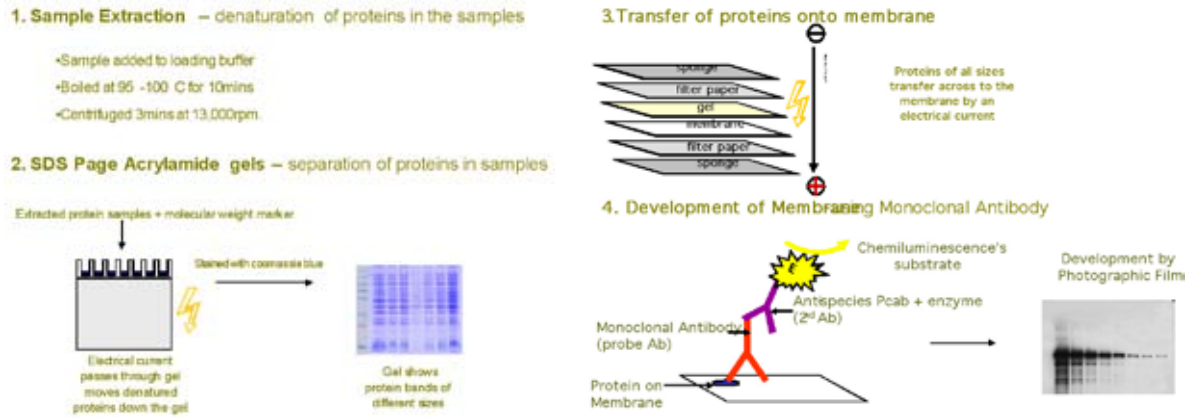
3.3 1-D and 2-D protein gel electrophoresis

One dimension (1-D) polyacrylamide gel electrophoresis (PAGE) and isoelectric focussing gels are used to differentiate proteins on the basis of charge mainly, but to some extent folding properties (see Figure 23a). It would therefore be difficult to differentiate a single amino acid change. However, the method may be able to detect truncated proteins.

Two dimension (2-D) electrophoresis has been used to screen for protein differences in GM compared to non-GM organisms with techniques such as difference gel electrophoresis (DiGE) being applicable to determine differences between protein profiles.

Electrophoresis also offers the opportunity to separate proteins prior to probing with an antibody raised to a targeted protein by western blotting (see Figure 23b). 2-D gels separate proteins on the basis of charge and size thus increasing the likelihood that differences may be detected. In western blots 1- or 2-D gel electrophoresis of proteins is followed by specific identification of the protein using antibody-based detection (see Figure 23b). This may be more accurate than 2-D electrophoresis as specific epitopes on the protein can be targeted.

Figure 23: Separation and detection of proteins using 1D PAGE electrophoresis and western blotting



3.4 Conclusions for protein-based methods

If the genetic modification is not expressed at the protein level, protein-based methods are obviously not applicable.

Application of protein-based methods will be only possible when the following prerequisites are fulfilled:

- Prior information on the new protein or on the protein modification/amino acid change is required to be able to apply protein-based methods.
- Protein-based methods require intact proteins in sufficient amount, so processing of the material reduces or completely excludes their applicability.
- The detection of a change in the protein would not always enable identification of a specific genetic modification. In general, a protein-based detection method will only be useful where the genetic modification creates a novel or changed protein (e.g. post-translational modification) or removes a protein product. It is anticipated that in most modifications this will be the case as the aim of the modification will be to change some function in the plant.

Immuno-based methods like Lateral Flow Devices (LFD) and Enzyme Linked Immuno Sorbent Assays (ELISA) are particularly useful for routine use in detection (and possibly identification) of genetic modifications but the development of the required antibodies involve some investment in research and development. Protein sequencing, electrophoresis and western blots are less useful for the analysis of many samples on a routine basis.

4 Metabolite-based analysis

Metabolites are substances produced by the metabolism of the plants. Metabolites encompass a wide range of chemical compounds. Primary metabolites are required to maintain the functioning of the cell for processes such as photosynthesis or respiration. Secondary metabolites have a function in the plant.

A process of genetic modification is expected to change the metabolite profile of an organism when compared to the wild-type. The metabolite pool from an organism is called the metabolome and its study is called metabolomics.

In metabolomic studies, differences in metabolomic profiles from different groups of organisms (e.g. GM and non-GM organisms) are ascertained. A statistically representative number of samples are analysed

using a non-targeted technique. Many different techniques can be used to perform these studies but the most powerful are those of Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS), hyphenated with either gas chromatography (GC-MS) or high performance liquid chromatography (LC-MS). Each technique has its advantages and these are detailed below.

4.1 Gas Chromatography in combination with Mass Spectrometry

Gas chromatography (GC) in combination with mass spectrometry (GC-MS) is one of the most frequently used tools for metabolomics. Instruments are now mature enough to run large sequences of samples; novel advancements increase the breadth of compounds that can be analyzed, and improved algorithms and databases are employed to capture and utilise biologically relevant information.

A mixture of compounds to be analysed is injected into the gas chromatograph where the mixture is vaporised. The gas mixture travels through a GC column, where the compounds are separated as they interact with the stationary phase on inner walls of the column and then enter the mass spectrometer. The achievable range and number of metabolites profiled by GC-MS can be attributed to the high separation efficiencies of long (30-60 m) capillary GC columns (i.e. N ≥ 250 000 for 60 m). These high efficiencies enable the separation of very complex mixtures. Recent developments include comprehensive GCxGC-MS, which separated compounds with two columns of orthogonal properties.

For successful GC, analytes have to be sufficiently volatile to be vaporised in the injector and to partition from the column back into the carrier gas. Plant metabolites such as sugars, amino acids, and hydroxy acids include many different chemical moieties, often present in the same molecule. As most of these compounds are not volatile, they have to be derivatised before GC analysis (typically silylating reagents).

In most cases GC-MS experiments are performed in electron ionisation (EI) mode with compound identification based on matching acquired spectra to mass spectral databases libraries. The versatility of large libraries like the NIST08 mass spectral resource lies in the fact that EI mass spectra are comparable over a wide range of different types of mass spectrometers from different vendors. In addition to mass spectral library searching and retention index-matching, a number of steps can be taken to interpret the mass spectrum, including accurate mass measurements by high-resolution mass spectrometry, study of isotope ratios, study of the neutral losses and tandem mass spectrometry (MS/MS).

Two orthogonal strategies are typically employed: metabolic profiling and targeted analysis.

Metabolic profiling (also known as differential expression analysis or discovery metabolomics) finds interesting metabolites with statistically significant variations in abundance within a set of experimental and control samples. The goal is to provide a more or less holistic study of a metabolome with detection of hundreds or thousands of metabolites. Although metabolic profiling has been described as unbiased and global, in reality all methods of sample preparation and all analytical platforms introduce a level of chemical bias. GC-MS has proven capability for profiling large numbers of metabolites with reports covering several hundred to slightly more than a thousand various components.

Targeted metabolomics may be used to validate hypothesises from the discovery step or investigate metabolic models focusing on specific known metabolites. The analytical requirements for these studies are different in that profiling relies on nonbiased, quantitative analysis of all or a large number of metabolites and so all the mass spectral data generated must be acquired, methods must cover a wide range of metabolites, most with low and high relative abundance. This challenge limits the scope of GC-MS instruments based on a single quadrupole analyser for metabolic profiling studies as the technology shows insufficient sensitivity and acquisition speed in when scanning the full mass range mode. The use of TOF technology provides an innovative approach to overcoming these draw backs. Such instruments can operate at very high repetition rates and between 20 and 500 spectra per second can be stored. For example, up to 1,000 individual metabolites could be retrieved from plant tissues using GC-TOF concomitant

with deconvolution software to identify individual compounds based on detection of model ions even in those cases where the individual mass spectra of two or more compounds overlap.

Atmospheric pressure ionisation interfaces for mass spectrometry such as ESI, remove the necessity for derivatisation. High (or ultra high) performance liquid chromatography (HPLC or UHPLC) is readily coupled to mass spectrometry to yield a powerful tool for targeted metabolic profiling and non-targeted metabolomics. It is generally more sensitive than LC-UV/Vis and yields more accurate quantitative data. However, not all compounds ionise to the same extent. This becomes a problem in global metabolic studies but not in targeted metabolic studies where all compounds of interest have similar chemical properties. HPLC and UHPLC are efficient separation techniques that can be used to resolve different groups of compounds, hydrophilic as well as hydrophobic, salts, acids, bases, etc. HPLC in its present form has different chromatographic modes that can be tailored to the separation of a specific class of compounds. These modes include reversed-phase (RP), normal phase, ion exchange, chiral, size exclusion, hydrophilic interaction chromatography (HILIC), and mixed modes. The popularity of RP columns (silica-based or monolithic) stems from their applicability to the majority of compounds and their simplicity and ease of use. Recent advances in column technology, such as HILIC, allow the detection of highly polar compounds, un-retained using RP systems. UHPLC introduced high chromatographic peak resolution to LC resulting in increased speed, sensitivity and peak capacity/coverage.

Metabolic profiling of biological samples results in a plethora of data that can be overwhelming in its abundance. For meaningful interpretation, the appropriate statistical tools must be employed to manipulate the large raw data sets in order to provide a useful, understandable, and workable format. Different multidimensional and multivariate statistical analyses and pattern recognition programs have been developed to distil the large amounts of data in an effort to interpret the complex metabolic pathway information from the measurements.

4.2 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a non-selective technique that can be tuned so that all soluble molecules containing 1H atoms will give an observable resonance peak (i.e. solution state 1H NMR spectroscopy). The NMR signal arises from the population difference between aligned nuclei within a magnetic field.

The NMR signals are presented on the chemical shift scale which is machine independent. Therefore, spectra acquired on one spectrometer can be directly compared to spectra generated on another, even at different magnetic field strengths. Chemical shift is dependent on the chemical structure and the local chemical environment of the molecule under observation. Further information about the chemical structure is inherent in the NMR spectrum as J couplings. The NMR measurement is therefore highly specific and well suited to discriminating between similar compounds (including isomers). Peak area is directly correlated to 1H concentration and therefore can be used to determine analyte concentration.

NMR spectroscopy is a particularly powerful technique in the area of metabolomics. When correctly implemented, NMR spectroscopy is a primary ratio method, i.e. a single internal standard can be used to quantify all analytes detected. Furthermore, separation is achieved from the intrinsic properties of the analytes and is therefore extremely reproducible. Data produced by NMR spectroscopy is ideally suited for subsequent statistical analysis. Where statistical analysis is able to ascertain differences between sample populations it can be related back to peaks in the NMR spectrum. These peaks can then be assigned by either database searching, or in the case of novel metabolites using advanced multidimensional NMR techniques.

4.3 Conclusions for metabolite-based methods

The most powerful of the metabolite-based techniques are Nuclear Magnetic Resonance (NMR), Gas Chromatography – Mass Spectrometry (GC-MS) and Liquid Chromatography – Mass Spectrometry (LC-MS). Each technique has its own merits. To ensure maximum coverage of metabolites, parallel studies implementing all techniques are advised. The strength of the techniques is in screening for unexpected effects.

Where significant differences are determined (either differences in concentrations of metabolites, or presence of novel metabolites) they form the basis of metabolite-based detection strategies. Once known, these differences can be determined using simpler analytical techniques so that more cost effective routine screening can be performed.

To use any of these techniques there would be a significant need for method development to make the techniques reproducible and non-selective. The techniques need to be: sensitive (MS better than NMR), reproducible (NMR better than MS), have the ability to elucidate structure (NMR and MS can both do this). Also there is a need to improve statistical analysis to find out which analytes are significant and robust biomarkers of differences.

However, metabolite-based methods alone would not be able to detect, identify or differentiate plants modified with a specific genetic modification technique from similar plants produced using a different technology. They may be used in combination with other techniques to detect or identify plants modified with a specific genetic modification technique.

5 General conclusions on detection and identification of genetic modifications

To date a broad range of methods can be applied to detect genetic modifications, including DNA-based methods, protein-based methods and metabolite analysis.

Based on the review of this large diversity of methodologies, the NTTF considers that:

- DNA is the ideal target molecule for detecting and identifying unambiguously a change in the genetic material of an organism as the intended result of the use of a genetic modification technique.

- DNA-based methods are the most appropriate for detection and identification of genetic modifications and offer potentially all required levels of specificity and ability to quantify the target i.e. a specific DNA sequence (protein-based methods or metabolite analysis methods have in particular some limitations in terms of identification of a change as the intended result of the use of a genetic modification technique and of differentiation with natural mutation).

- Within DNA-based methods, DNA amplification-based methods (PCR) are nowadays the most appropriate for detection and identification of genetic modifications (DNA-sequencing methods have in particular some limitations in terms of practical application for routine analysis while DNA-hybridisation methods have some limitations in terms of sensitivity).

However, any PCR-based method relies on the availability of a certain minimum of information about the target DNA sequence. Some information needs to be known about the inserted DNA sequence and about the 5‘ and/or 3‘ neighbouring genomic DNA sequence in order to allow the identification of an intentional genetic modification (see further details below). Without prior knowledge, reliable identification of a genetic modification is not possible even with the most sophisticated available methods for DNA analysis.

Part 2: Specific considerations for detection and identification of intentional genetic modifications by new plant breeding techniques

Based on the previous section the NTTF comes to the general conclusion that DNA amplification-based methods (PCR) are the most appropriate for detection and identification of genetic modifications.

The EU regulatory approach based on validation of GMO event-specific PCR methods can be considered as the "reference" or "baseline" for detection and identification of products obtained through a deliberate genetic modification technique, be it through genetic engineering (like GMOs defined under Article 2 (2) in conjunction with Annex IA Part 1 of Directive 2001/18/EC) or through a new technique.

For each GMO to be approved in the EU, detailed information on molecular characterisation and detection of the specific GMO is to be provided by the applicant as part of the EU GMO regulatory approval process. Accordingly, a PCR-based event-specific detection method is validated by the EU Reference Laboratory for GM Food Feed before any GMO can be approved in the EU (detailed information on the activities of the EU Reference Laboratory for GM Food Feed and the information to be provided by applicants about GMO detection and identification method (incl. list and protocols of validated detection methods) is available at http://gmo-crl.jrc.ec.europa.eu/default.htm)

In this section we report the possibilities of detection and identification for each of the seven individual new plant breeding techniques. Based on current available detection methods summarised before, the "reference" or "baseline" for this analysis was therefore the PCR-based approach for detection of GMOs (known or unknown).

- Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
- Oligonucleotide directed mutagenesis (ODM)
- Cisgenesis and intragenesis
- RNA-dependent DNA methylation (RdDM)
- Grafting (on GM rootstock)
- Reverse breeding
- Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

For each specific new plant breeding technique the following information is given:

1. Definition of the individual New Technique (including if need be some general considerations)

For consistency reasons, the NTTF agreed to use definitions of the above new plant breeding techniques which are in line with the ones used in the draft report from the NTWG (where further details on the definitions, rationale for use in plant breeding and mechanism of each individual New Technique can be found)

2. Detection and identification with prior knowledge

This scenario refers to cases where information is available (in particular at the level of DNA sequence) on the product resulting from the use of a new plant breeding technique. This information may be made available for instance from the company having developed the new product (plant).

Cross-reference is made to Chapter 7.1 which includes details on the type of information required to allow detection and identification of genetic modification.

3. Detection and identification without prior knowledge

This scenario refers to cases where no information at all is available on the product resulting from the use of a new technique.

It is to be noted that in the case of "unknown" GMOs (i.e. GMOs for which no information is available for instance because no regulatory application has been filed) detection and identification are challenging. For detection of unknown GMOs, the usual detection approach is to use PCR-methods to screen for certain genetic elements which are commonly present in GMOs (like the 35S promoter or the nos terminator). However, this screening approach does not allow detection of all GMOs and anyway does not allow identification of a specific GMO event.

Note: a new document from the ENGL on "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials" is under preparation and is expected to be published in the first quarter of 2011. This upcoming ENGL publication will provide further detailed information on the challenges raised by the detection of "unknown" GMOs, which may be relevant to the ones raised in the present report under the scenario "Without prior knowledge".

4. Conclusions

The conclusions summarise the opinion of the NTTF regarding the possibility to detect and more importantly to identify products from the various individual new plant breeding techniques i.e. the possibility to differentiate them from products resulting from natural mutations or obtained from other breeding techniques, e.g. mutagenesis.

1. Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)

1.1 Definition

Zinc Finger Nuclease (ZFN) technology is a highly specific DNA targeting tool allowing specific changes of nucleotide sequence. ZFN technology is based on the use of zinc-finger nucleases which are hybrid proteins combining a non-specific DNA cleavage domain of the *Fok*I restriction enzyme and a specific DNA binding domain with several C_2H_2 zinc-fingers for cleavage specificity (Zinc Finger domains can be custom-designed to bind to a specific site within a given locus thereby providing a highly specific targeting tool). In the cell, the ZFN complex recognises the target DNA site and generates a double strand break at a specific genomic location. This stimulates native cellular repair processes: homologous recombination and non-homologous end-joining, thus facilitating site-specific mutagenesis.

In line with the options considered by the NTWG, three different ways of using ZFN technology have been analysed by the NTTF:

ZFN-1: generates site-specific random mutations (short deletions or/and insertions, changes of single base pairs) by non-homologous end-joining. No repair template is provided. In case of short insertions the inserted material is from the organism's own genome.

ZFN-2: uses a short repair template to introduce site-specific changes in nucleotide sequence (short deletions or/and insertions, specific nucleotide substitutions of a single or a few nucleotides) by homologous recombination. The repair template is delivered to the cells simultaneously with the ZFN.

ZFN-3: allows insertions of entire genes at specific locations. DNA fragments of up to several kilo base pairs (kbp) are introduced together with ZFNs. Site-specific insertion, removal, replacement and/or stacking of larger genetic elements occurs by homologous recombination.

At present, genes from ZFN complex are delivered by electroporation, viral vectors or *Agrobacterium* mediated transfer. If the constructs are not replicated or integrated, their presence is transient and they can not be detected in products. In the future, ZFNs may be delivered directly as proteins.

At present, DNA-based methods are therefore the most appropriate for detection and identification of ZFN products.

1.2 Detection and identification with prior knowledge

ZFN-1 and ZFN-2

In the case of ZFN-1 and ZFN-2, the introduced genetic modifications correspond to small modifications (a single or few nucleotides). For detection of small changes in DNA, DNA-based detection methods are the primary approach and amplification based methods (PCR) already exist for the detection of short insertion, deletions (see part 1 Chapter 2.1).

Provided information is available (nucleotide sequence of approximately 20 bp including the modification and its immediate vicinity), detection of ZFN-1 or ZFN-2 modification is possible. However identification is not possible because ZFN-1 and ZFN-2 products cannot be distinguished at molecular level from those developed through other mutation techniques (using chemicals or ionising radiations) or occurring through spontaneous natural mutations.

ZFN-3

In the case of ZFN-3, the introduced genetic modifications correspond to large modifications (several kbp). The amplification based methods (PCR) presently used for the detection of GMOs are available to detect and also to identify the products as resulting from the use of the ZFN-3 technique.

1.3 Detection and identification without prior knowledge

ZFN-1 and ZFN-2

Without prior knowledge of DNA sequence, amplification-based methods like PCR cannot be used. Analysis of whole genome through DNA sequencing could in theory be used to possibly detect some short insertions and deletions. However this would be a burdensome approach which cannot be used on a routine basis. It will anyway not allow to identify ZFN-1 and ZFN-2 products and to differentiate them from products from natural mutations or other mutation techniques.

ZFN-3

In the absence of DNA sequence information, the detection of large modifications that are the results of ZFN-3 technology methods would present challenges similar to the ones which are currently used for detection of unknown GMOs. Identification of products from ZFN-3 will not be possible without any prior knowledge.

1.4 Conclusion

ZFN-1 and ZFN-2

For organisms modified by the ZFN-1 and ZFN-2 techniques (leading to small modifications) detection with DNA based methods would be possible provided some prior information on the introduced modification is available. But identification will not be possible because ZFN-1 and ZFN-2 products cannot be distinguished at molecular level from products developed through other mutation techniques or occurring through natural mutations (see Chapter 7.1 Modification of one or a few nucleotides).

Without prior knowledge, detection of small modifications introduced by ZFN-1 and ZFN-2 would be demanding and unlikely to be used in routine laboratories. Identification will not be possible.

ZFN-3

Detection and identification of organisms modified by ZFN-3 technology (leading to large modifications) is possible through the amplification based methods (PCR) currently used for GMO detection, with the prerequisite that prior adequate DNA sequence information on the introduced modification is available (see Chapter 7.1 Insertions larger than 80 bp).

If there is no prior knowledge, the strategies used for detection of unknown GMOs may be applied to detect the large modifications resulting from ZFN-3. Identification will however not be possible without prior knowledge.

2. Oligonucleotide directed mutagenesis (ODM)

2.1 Definition

The oligonucleotide directed mutagenesis (ODM) employs oligonucleotides for targeted (site-specific) changes of one or a few adjacent nucleotides. ODM allows the correction or introduction of specific mutations (base substitution, insertion or deletion) at defined sites of the genome by using chemically synthesised oligonucleotides.

ODM makes use of different types of oligonucleotides of approximately 20 to 100 nucleotides with homology to the target gene (except for the nucleotide(s) to be changed). Examples are single-stranded DNA oligonucleotides containing 5' and/or 3' modified ends to protect the molecule against cellular nuclease activities, chimeric RNA/DNA or DNA/DNA, RNA oligonucleotides, and triplexforming oligonucleotides.

Using ODM only one to maximum four adjacent nucleotides will be modified.

The gene modification is induced directly and exclusively via the effect of the oligonucleotide itself, i.e. independent of a vector system. Therefore, ODM does not involve the introduction or integration of foreign DNA.

2.2 Detection and identification with prior knowledge

DNA-based methods are the primary techniques to be used for the detection of the mutations which are the result of ODM. For the detection of ODM products, knowledge of the nucleotides in the vicinity of the introduced mutation is necessary to be able to design primers (as detailed in part 1 Chapter 2).

However DNA-amplification-methods using primers that encompasses the mutation would not be sufficiently reliable as a lack of specificity of the primers may give false positives or negatives. DNA-sequence analysis will also need to be used in combination to allow the detection of ODM products.

The identification of the results of ODM will anyway not be possible as these kinds of mutations can not be differentiated at the molecular level from those developed through other mutation techniques (chemical or radiation mutagenesis) or naturally occurring mutations.

In theory, protein-based detection methods may be used provided the targeted mutation results in an alteration at the protein level (change in amino acid sequence). Like for other new plant breeding techniques, amino acid sequencing or methods based on the detection of altered physicochemical characteristics of the protein (e.g. folding properties, charge, altered binding properties to antibodies due to altered epitopes) may allow the detection of ODM products (not their identification) but these techniques are in any case not applicable for routine analysis.

2.3 Detection and identification without prior knowledge

In the absence of any prior knowledge, DNA-amplification based methods cannot be used (see part 1 Chapter 2).

In some cases of ODM, phenotype differences compared to natural variants may give an indication of the locus of the mutation.

In any case identification of ODM products will not be possible as the presence of natural mutations (for instance spontaneous mutation occurring during breeding process or single nucleotide polymorphism) could potentially mimic the targeted mutations.

2.4 Conclusion

Mutations that are the result of ODM can be detected by PCR-based methods as long as certain information on the nucleotides in the vicinity of the mutation is known. This is necessary to be able to design primers. Without such information, the mutation cannot even be detected.

In any case, methods allowing the detection of mutations do not allow identification of ODM products.

It is not possible to distinguish at the molecular level organisms developed through ODM from organisms bearing the same mutation obtained through other mutation techniques (chemical or radiation mutagenesis). It is also not possible to differentiate ODM products from spontaneous mutations or single nucleotide polymorphism mutations (see Chapter 7.1 Modification of a few nucleotides).

3. Cisgenesis and intragenesis

3.1 Definition

Cisgenesis is a genetic modification of a recipient species with a natural gene from a crossable - sexually compatible – organism (same species or closely related species). Such a gene includes its introns and is flanked by a native promoter and terminator in the normal sense orientation. Where different fragments from the same organism are combined, the technique result is defined as intragenesis.

Intragenesis is different from cisgenesis. This is the integration of an intragene. An intragene is commonly a hybrid gene and intragenesis involves the insertion of a reorganised, full or partial coding part of a natural gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species.

Cisgenic plants can harbour one or more cisgenes, but they do not contain any transgenes. To produce cisgenic plants any suitable technique used for production of genetically modified organisms may be used. Genes must be isolated, cloned and transformed back into a recipient.

Next to the definition mentioned above, there is an additional NTWG prerequisite that the cisgenic plant should not contain any foreign DNA: “In the case of transformation via Agrobacterium tumefaciens it must be demonstrated that no border sequences are inserted along with the gene. Where border DNA or any foreign DNA is inserted, the technique is not considered as cisgenesis or intragenesis and the resulting organism is a GMO according to the Directives.”

In the discussion below, cisgenesis and intragenesis will be discussed separately.

In some applications of cisgenesis, it is envisaged that a selection marker will be used to screen for primary transformants. The selection marker is then removed in a later stage. This could result is a residual border trace. Furthermore, a transformation with *A. tumefaciens* leaves in most cases a residual T-DNA border trace.

3.2 Detection and identification with prior knowledge

Cisgenesis

Detection with the current techniques (primarily with qPCR on DNA level) is feasible if the producer provides information on the transformation event that took place to enable the cisgenic insertion.

Identification is also possible provided adequate information is provided by the producer (see part 1 Chapter 2.1 - DNA sequence information on the insertion introduced by genetic modification and on the neighbouring genomic DNA).

Products similar to the cisgenesis ones may be obtained through conventional breeding. Nevertheless identification of products obtained by cisgenesis is still possible due to the unique event-specific transition in nucleotide sequence: although no novel material (i.e. present only outside the species' gene pool) was added, the rearrangement that took place to insert the transformation cassette into the host organism has a distinct character that can be visualised by event-specific primers/probe.

Intragenesis

For intragenic plants, the detection and identification possibilities are analogous to cisgenic plants i.e. both detection and identification are possible provided adequate information is made available (see part 1 Chapter 2.1 - DNA sequence information on the insertion introduced by genetic modification and on the neighbouring genomic DNA).

Note: the producer should provide positive reference material and negative control material to allow a detection method that can be validated.

3.3 Detection and identification without prior knowledge

Cisgenesis

Due to the intrinsic properties of a cisgenic plant (i.e. that the inserted property consists of only material from within the species' gene pool without any DNA from outside the species' gene pool), it is not possible to screen for a certain common element (like the 35S promoter is for instance used in screening for unknown GMOs).

The detection of plants that were established by a cisgenic approach might theoretically be achieved by sequencing: in the case were some information is present on the introduced sequence, it is possible to sequence outward from the known nucleotide sequence. However such detection approach would be part of a research project, and can not be part of a routine analysis due to the extensive experiments required.

In addition the modification resulting from cisgenesis cannot be identified as such without prior knowledge from the producer. A genome analysis by means of transcriptome sequencing or even whole genome sequencing could possibly detect the insert, although the success rate is unknown. The prerequisites are the presence a pure reference material and knowledge on the comparators that can be used as a baseline, although the sequencing process is not easy.

Intragenesis

For intragenic plants, the possibilities for detection are analogous to cisgenic plants. However, with intragenic (re)shuffling it would theoretically be more obvious that a certain rearrangement in a gene would be the result of intragenesis than that it would be caused by natural rearrangement of the genome.

3.4 Conclusion

Cisgenic/intragenic plants harbour genes that were derived from within the gene pool of the same species.

Cisgenic/intragenic plants can be detected and identified as such when the event is known beforehand i.e. when adequate information about the cisgenesis/intragenesis modification is made available (see Chapter 7.1 Insertions larger than 80 bp). Event-specific primers can be developed to create a detection and identification method.

In the case of unknown alterations, sequencing (genome or transcriptome) could in theory support the detection of plants but the method has not been validated yet for this purpose. Therefore it can be concluded that without prior knowledge, the detection and the identification of cisgenic and intragenic plants is not feasible at this moment.

4. RNA-dependent DNA methylation

4.1 Definition

The RNA-dependent DNA methylation technique (RdDM) utilises small RNA – miRNA (micro RNA) or siRNA (small interfering RNA) to inhibit gene expression by methylation of the DNA. Gene silencing via DNA methylation can be accomplished in an organism by transfection of the cells with genes coding for RNAs which once transcribed, give rise to the formation of small double stranded RNAs (interfering RNAs). If these double stranded RNA molecules share homology with sequences in the organism's DNA (e.g. a promoter region) they can specifically induce/guide methylation resulting in the silencing of the downstream genes. The sequence of the inserted gene (which will be homologous to the gene of interest) will determine the specific target for DNA methylation and thus for gene silencing. Therefore RdDM allows highly selective gene silencing.

As a general consideration, it should be noted that the knowledge on gene silencing and regulation of gene expression by methylation is still rather limited and it is very difficult to differentiate methylation processes occurring naturally and through the deliberate use of a genetic modification technique. In addition methylation can also be detected in non-silenced genes (it is the density of methylation which has an impact on the phenotype).

4.2 Detection and identification with prior knowledge

In theory, different options may be considered for the detection of RdDM products.

A first approach would be methods that allow monitoring of gene expression (namely reverse-transcription coupled with real-time quantitative PCR – RT qPCR). These may be performed by control laboratories as the equipment is the same as routine GMO analysis. However, full validation of such methods should precede and suitable references would need to be developed. This approach is anyway applicable only in case of non-processed material, where RNA is intact. It is also important to keep in mind that when the template RNA for double stranded RNA is introduced by transfection or by a vector system, the templates are intended to be present only transiently in the cell and are expected to be absent from the final commercialised

product. When an RNAi construct is used, commercial products lacking the construct can be obtained by segregation. In all cases a screening procedure to test for the absence of this construct would be a logical part of the selection process.

There are also several methods for the analysis of DNA-methylation status at individual loci including:

Methylation specific PCR-based techniques based on amplification of bisulphite-converted DNA. These techniques can detect the presence of specific DNA patterns with very high sensitivity and specificity.

Methylation-sensitive/dependent restriction enzymes. Principle of methylation-sensitive restriction technique is that the methylation-sensitive restriction enzymes cannot cut the methylated DNA site.

Methylation-Sensitive High-Resolution Melting (MS-HRM) analysis. High-resolution melting (HRM) analysis exploits the reduced thermal stability of DNA fragments that contain base mismatches to detect single nucleotide polymorphisms (SNPs). High Resolution Melting (HRM) relies upon on the precise monitoring of the change of fluorescence as a DNA duplex melts. Like many real-time PCR techniques, HRM utilizes the ability of certain dyes to fluoresce when intercalated with double-stranded DNA. Methylated DNA has enhanced thermal stability and is sufficiently divergent from non-methylated DNA to allow detection and quantification by HRM analysis. This approach reliably distinguishes between sequence-identical DNA differing only in the methylation of one base. By comparing the melting profiles of unknown samples with the profiles of fully methylated and unmethylated references amplified after bisulphite modification, it is possible to detect methylation with high sensitivity and moreover estimate the extent of methylation of the screened samples.

Various options may in theory be available for detection of RdDM products but further work on validation of these methods would still be required before they could be used.

In addition, according to the current state of knowledge, it is extremely difficult to differentiate between organisms resulting from the deliberate use of a plant breeding technique like RdDM technique and organisms resulting from methylation processes occurring naturally.

It can therefore be concluded that identification of RdDM products is not possible, even with prior knowledge.

4.3 Detection and identification without prior knowledge

Methylation status at individual loci in plant genomes under different developmental or environmental conditions is not available. Only some information is known on *Arabidopsis thaliana*, the model species.

A theoretical option for detecting “unknown” RdM products may be whole genome DNA methylation analyses. Current standard procedures involve complete enzymatic hydrolysis of DNA, followed by high-resolution separation to obtain the total base composition of the genome. However it should be stressed that this is not yet a routine technique that can be commonly used in laboratories. In addition it is to be noted that such methods are not validated, that results would require comprehensive bioinformatics processing and that suitable comparators are not available.

It can therefore be concluded that without prior knowledge identification of RdDM products is not possible.

4.4 Conclusion

Specific gene silencing is obtained through DNA methylation and/or histone methylation in the chromatin but the DNA sequence itself is not modified.

Since it is very difficult to differentiate between methylation occurring naturally and methylation through the deliberate use of a technique like RdDM, it can be concluded that identification of RdDM products is not possible, even with prior knowledge.

5. Grafting (on GM rootstock)

5.1 Definition

Grafting is a technique used to combine desired traits of the rootstock with those of the donor plant shoot, or scion. It is a method whereby a vegetative top part (the graft or scion) of one plant is attached to a rooted lower part (the rootstock) of another plant.

Two possibilities can be considered:

Grafting a non-GM scion onto a GM rootstock

Grafting a GM scion onto a non-GM rootstock

In practice however grafting on a transgenic rootstock that is beneficial for the scion, e.g. flowers or fruit, is the most common example of grafting. Most commercial applications will likely focus on a GM rootstock and a non-GM scion since the harvested product (fruit, flowers etc.) is above ground.

Grafting of a non-GM scion onto a GM rootstock is therefore the case on which the NTTF focused.

Note: it is also possible to graft a GM scion onto a GM rootstock. This will result in a full chimaeric GM plant and was therefore not considered in the present report.

An important general consideration to stress is that until now, no scientific evidence has been pointing toward a transfer of the GM-derived DNA into the scion. Therefore, it will be very difficult, or even impossible, to detect the GM moiety in the harvested product.

5.2 Detection and identification with prior knowledge

It is virtually impossible to design a DNA-based strategy in order to detect or to identify non-GM scions (and products harvested from the scion) that were grafted on GM-rootstocks.

If the whole chimaeric plant is regarded (including the GM rootstock), it will be possible to detect and identify it with PCR-methods like a “regular” GMO as defined in Annex IA of Directive 2001/18/EC.

Note: RNA molecules, proteins and metabolites that are related to the genetic modification may be transported from the GM rootstock to the non-GM scion. Alternative methods to DNA-based methods may be transcriptome analysis, which visualises the different transcripts (present/absent, and the respective level). If the harvested product was originating from a scion that was grafted on a GM-rootstock, it can be expected that the scion has a deviating transcriptome compared to the case in which it was grafted on a non-GM rootstock. The prerequisites will however be difficult to establish, and the method has not been validated yet. This may be part of a research project but cannot be done as a routine analysis.

5.3 Detection and identification without prior knowledge

It is virtually impossible to design a DNA-based strategy to be able to identify harvested products from non-GM scions that were grafted on GM-rootstocks.

5.4 Conclusion

Grafting of a non-GM scion onto a GM rootstock is the case on which the NTTF focused.

As the DNA sequence of the non-GM scion is not modified, detection and identification of the GM rootstock on the basis of the harvested product (part of the non-GM scion) is not possible today and is very unlikely to be developed in the near future.

6. Reverse breeding

6.1 Definition

Reverse breeding is a new plant breeding technique that aims to produce parental lines to be used for reconstruction of any heterozygous plant.

Homozygous parental lines are produced from selected heterozygous plants by suppressing meiotic recombination. This suppression is obtained through RNAi-mediated down-regulation of genes involved in the meiotic recombination process.

As a result, the haploid gametes of the genetically modified plant contain entirely non-recombinated chromosomes. These gametes are subsequently used to produce double haploid plants (DH) by *in vitro* regeneration. Double haploid plants are screened for the absence of the RNAi construct before they are crossed to the complementary parent to obtain the hybrid variety.

During the breeding the genes used for the genetic modification are crossed out resulting in end-products that are completely free of genetic modification-related RNAi constructs. The reconstructed hybrid variety is the final commercial product.

6.2 Detection and identification with prior knowledge

In some cases gene silencing using RNAi can lead to RNA-directed DNA methylation of the transcribed region. In such cases, like for the RdDM technique (see Chapter 4), the following methods may be used for potential detection of methylation-related changes:

Methylation specific PCR-based techniques based on amplification of bisulphite-converted DNA

Methylation-sensitive/dependent restriction enzymes. Principle of methylation-sensitive restriction technique is that the methylation-sensitive restriction enzymes cannot cut the methylated DNA site

Methylation-Sensitive High-Resolution Melting (MS-HRM) analysis. High-resolution melting (HRM) analysis exploits the reduced thermal stability of DNA fragments that contain base mismatches to detect single nucleotide polymorphisms (SNPs)

However, like in the case of the RdDM technique (see chapter 4), it will in any case not be possible to identify the source of DNA methylation as resulting from a specific plant breeding technique since the DNA-methylation phenomenon also occurs in nature.

Note: standard PCR techniques are suitable to reliably confirm the absence of genetic modification-related DNA sequences into the lines selected for further breeding.

6.3 Detection and identification without prior knowledge

Both detection and identification are not possible.

6.4 Conclusion

The end-products of reverse breeding are free of genetic modification-related DNA sequences since the homozygous parental lines are produced from double-haploid plants which are screened for the absence of RNAi construct during the breeding process.

It is therefore not possible to distinguish products resulting from the use of reverse breeding technique from products resulting from conventional breeding. Identification of products resulting from the use of reverse breeding technique is therefore not possible.

7. Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)

7.1 Definition

Plant tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium sp.* containing a foreign genetic construct. This genetic construct is locally expressed at high level. Other terms often used in this context are agro-infection, agro-inoculation.

In most of the cases these technologies are carried out on non-germline plant tissues. The result is transient expression of the genes introduced in the plant cells.

An exception is flower dip where germline tissue is infiltrated with *Agrobacterium* with the aim to obtain stably transformed seedlings.

Depending on the tissues and the type of constructs infiltrated, three types of agro-infiltration can be distinguished (like it was done in the NTWG):

"Agro-infiltration sensu stricto":

Non-germline tissues are infiltrated with non-replicative constructs in order to obtain localised expression in the infiltrated area. Agro-infiltration is a screening tool carried out on detached plant parts or on intact plants. In principle after the observations the infiltrated plants will be destroyed and a clone with the identified desired phenotype will be used for further breeding. The resulting products, e.g. a new cultivar, will not contain the infiltrated DNA fragments, and therefore cannot be detected as a cultivar being the result of a breeding strategy in which agro-infiltration has been used.

"Agro-inoculation" or "agro-infection":

Non-germline tissues (typically leaf tissues) are infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant.

"Floral dip":

Germline tissues (typically, flowers) are infiltrated with a DNA-construct in order to obtain transformation of embryos that can be selected during the germination phase. The aim is to obtain stably transformed plants, and therefore the resulting plants are genetically modified plants.

7.2 Detection and identification with prior knowledge

"Agro-infiltration sensu stricto":

During the experimental phase, transiently present DNA fragments can be detected by means of DNA based methods such as PCR. Primers for the PCR reaction are based on the sequence of the DNA fragments used for the agro-infiltration.

Transient expression has also been developed as a production platform for high value recombinant proteins. The approach can result in a high yield of the end product. In this case, the plant of interest is the agro-infiltrated plant and not its progeny. Detection of recombinant proteins is possible using standard protein based detection methods that can be immune based assays such as ELISA or chemical analytical tools such as amino acid sequencing or mass spectrometry based methods. But in case the recombinant protein is not different from the natural one no distinction is possible.

Transfer of T-DNA or DNA in general into the plant cell genome occurs only with a very low frequency. It is theoretically possible for the injected bacteria and DNA to spread through the plant and possibly transform cells elsewhere. The chance that by inoculating vegetative tissue this leads to the regeneration of a GMO offspring is extremely low. But in case it occurs detection is possible using the technologies that are currently used for GMO detection and identification, based on the information on the DNA constructs used in the agro-infiltration experiment.

"Agro-inoculation" or "agro-infection":

Idem as for 1.

"Floral dip":

The aim of floral dip is the selection and propagation of plants with stably inserted DNA fragments. These plants can therefore be detected and identified by using the technologies that are currently used for GMO detection and identification.

7.3 Detection and identification without prior knowledge

"Agro-infiltration sensu stricto":

In the primary transformant, the strategy will be identical as the one applied for the detection of unknown GMOs. The first step will be based on a DNA based screening strategy that can be complemented by information technology to enrich for potential positive samples to be analysed and to select DNA fragments that are known to be used in the context of agro-infiltration and might potentially be present.

In the genetic offspring from the infiltrated plant, the T-DNA was not inserted in the germline and is therefore not present in the progeny.

"Agro-inoculation" or "agro-infection":

Idem as for 1.

"Floral dip":

The strategy to detect products that are the result of floral dip but for which no molecular data are available will be identical as for the detection of unknown GMOs. The first step will be based on screening.

7.4 Conclusion

If the constructs introduced into plants by agro-infiltration are not replicated and/or integrated, their presence is transient and can be detected only in the agro-infiltrated plant itself. These DNA fragments will not be transferred to the next generation so they can neither be detected nor identified in the progeny plant and the products derived thereof. Detection and identification of products from agro-infiltration or from agro-inoculation is therefore not possible.

Note: detection and identification of agro-infiltrated plants and progeny plants that contain stably inserted fragments is possible with the same methodologies that are currently developed and used for GMO detection, which also implies that adequate information needs to be available.

In the case of floral dip, it is the aim to select for stable integration into the germline, leading to a genetically modified plant, which means that detection and identification are possible with the methods currently available for GMO detection (PCR), and also implies that adequate information needs to be available.

If no prior information is available, identification will not be possible in any case.

Conclusions on identification of new plant breeding techniques:

The following conclusions were agreed by the NTTF for each individual new plant breeding technique. They have been grouped together in a NTTF Summary Table attached to the present NTTF report.

It is *not possible to identify* products from the following new plant breeding techniques (mainly because they cannot be differentiated from products obtained with conventional breeding products, with other mutation techniques (chemical or radiation mutagenesis) or through natural mutations):

1. Zinc finger nuclease technology 1 and 2
2. Oligonucleotide directed mutagenesis (ODM)
3. RNA-dependent DNA methylation (RdDM)
4. Grafting on a GM rootstock
5. Reverse breeding
6. Agro-infiltration (agro-infiltration and agro-inoculation)

It is possible to identify products from the following new plant breeding techniques, provided some prior information is available (about the DNA sequence introduced by the genetic modification and the neighbouring genomic DNA sequence):

1. Zinc finger nuclease technology 3
2. Cisgenesis and intragenesis
3. Agro-infiltration (floral dip)

Without any prior knowledge about the genetic modification introduced by a specific new plant breeding technique, it is not possible to identify products from this new technique.

NTTF OUTCOME SUMMARY TABLE

New Plant Breeding Technique Name	New Plant Breeding Technique Description	Identification Possibility With Prior Knowledge	Identification Possibility Without Prior Knowledge	Information Requirements for Identification	Identification Methods	Comments
Zinc finger nuclease (ZFN) technology						
ZFN-1	Genes encoding ZFNs are delivered without a repair template. The ZFN generates a site-specific double strand break. The natural DNA-repair process leads to (short) site-specific mutations (change, deletion or insertion of one or few bp)	NO	NO	See part 1 chapter 2 - the reference/baseline for identification of a genetic modification is the PCR-method approach used for GMO detection - a minimum of information about the target DNA sequence needs to be available (DNA sequence introduced by genetic modification and neighbouring genomic DNA sequence)	PCR	No differentiation possible with products from mutation techniques (chemical, radiation mutagenesis) or natural mutations
ZFN-2	Same as ZFN-1 but genes encoding ZFNs are delivered together with a short DNA repair template (one or few bp), which generate site-specific mutations through homologous recombination.	NO	NO	Idem	PCR	Idem
ZFN-3	Genes encoding ZFNs are delivered together with a long DNA stretch (several kbp), which is inserted in the genome in a site-specific manner	YES	NO	Idem	PCR	
Oligonucleotide directed mutagenesis (ODM)	Oligonucleotides target homologous DNA and induce site-specific nucleotide substitutions, insertions or deletions through gene repair mechanisms	NO	NO	Idem	PCR	Products do not contain any genetic material used for initial genetic modification - No differentiation possible with products from mutation techniques (chemical, radiation mutagenesis) or natural mutations

New Plant Breeding Technique Name	New Plant Breeding Technique Description	Identification Possibility With Prior Knowledge	Identification Possibility Without Prior Knowledge	Information Requirements for Identification	Identification Methods	Comments
Cisgenesis	A DNA fragment from the plant species itself or from a cross-compatible plant species is inserted into the plant genome.			Idem		
Cisgenesis	In the case of cisgenesis, the inserted gene is unchanged and includes its own introns and regulatory sequences.	YES	NO		PCR	
Intragenesis	In the case of intragenesis, the inserted DNA is a new combination of DNA fragments from the species itself or from a cross-compatible plant species.	YES	NO		PCR	
RNA-dependent DNA methylation (RdDM)	Genes encoding RNAs homologous to plant sequences are delivered. The formed RNAs induce methylation of the homologous sequences and inhibit their transcription.	NO	NO	Idem	PCR	RNA template absent from the end product - No differentiation possible with products from conventional breeding or natural mutations
Grafting (on GM rootstock)	The upper vegetative component of one plant (the scion) is attached to the lower rooted component of another plant (the rootstock) to produce a chimeric organism. Case considered is a non-GM scion grafted on a GM rootstock (most likely case to be used)	NO	NO	Idem	PCR	Products harvested from the non-GM scion do not contain any new genetic material (DNA) from the GM rootstock
Reverse Breeding	Homozygous parental lines are produced from selected heterozygous plants by suppressing meiotic recombination (through RNAi down-regulation). Subsequently Double Haploid lines are produced and hybridised.	NO	NO	Idem	PCR	End products do not contain any genetic material used for initial genetic modification (double haploid plants are screened for the absence of RNAi construct during the breeding selection) - No differentiation possible with conventional breeding products

Note: identification of a genetic modification means that it is possible not only to detect the existence of a change in the genetic material of an organism but it is also possible to identify the genetic modification as intentionally introduced by a new technique.

New Plant Breeding Technique Name	New Plant Breeding Technique Description	Identification Possibility With Prior Knowledge	Identification Possibility Without Prior Knowledge	Information Requirements for Identification	Identification Methods	Comments
Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip)				Idem	PCR	
Agro-infiltration	Non-germline tissues (typically leaves) are infiltrated with non-replicative constructs in order to obtain localised expression in the infiltrated area (no integration of genetic construct in plant genome)	NO	NO			If the constructs introduced by agro-infiltration are not replicated and/or integrated, their presence is transient and can be detected only in the agro-infiltrated plant itself. These DNA fragments will not be transferred to the next generation. No differentiation possible with conventional breeding products.
Agro-inoculation	Non-germline tissues (typically leaves) are infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant (no integration of genetic construct in plant genome)	NO	NO			Idem as agro-infiltration
Floral dip	Germline tissues (typically flowers) are infiltrated with a DNA-construct in order to obtain transformation of embryos that can be selected during the germination phase. The aim is to obtain stably transformed plants	YES	NO			

ANNEX 17: REFERENCES

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