NOVEL PLANT BREEDING TECHNIQUES

Consequences of new genetic modification-based plant breeding techniques in comparison to conventional plant breeding

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Dr. JG Schaart, Prof. Dr. RGF Visser
Wageningen UR Plant Breeding
Wageningen University and Research Center

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Executive summary

This report describes consequences of new plant breeding techniques for the environment and food and feed safety. The new plant breeding techniques considered in this report share as common feature that they all make use of a genetic modification step, somewhere in the production of improved plant lines. The aim of this genetic modification is to test plants for specific characteristics, to facilitate breeding, to add genes or alleles that have been isolated from the same species or to make small changes to native genes. Because of the involvement of a genetic modification step, all these techniques fall under the European Directive 2001/18/EC.

One common feature of the new plant breeding techniques is that they all lead to end products (plants or plant parts) that are free of genes that are foreign to the species. So, in the end only genes that were already part of the gene-pool of the species will be present in its genome. This means that end products of the new breeding techniques, in principle also can be achieved using conventional plant breeding techniques, but usually in a much longer time frame or with much more difficulties.

In Europe, the cultivation, trade and use of food and feed of any genetically modified crop is subject to EU regulations. A safety assessment of the genetically modified crop is part of the admission procedure. This assessment for environmental, food and feed safety is a time-consuming and costly (on average €6.8 million for a full assessment (EU)) procedure. Although the new breeding techniques have great potential for rapid improvement of crop species, the required safety assessment may obstruct the development of such new crops. This is especially the case for ‘small’ or ‘orphan’ crops like many vegetable, fruit and ornamental crops.

To be able to bring the new breeding techniques to practice, there is a great demand for modernization of the EU regulations for genetically modified organisms. This report describes a technical-scientific approach to assess possible consequences of new breeding techniques for the environment and human & animal health, and provides information that is important for consideration of adaptation of the EU regulations. For the discussion of these consequences, the new breeding techniques are compared to a baseline. The baseline is a reference, for example a similar plant, but bred according to conventional breeding techniques. The baseline covers the ‘natural’ situation in its full bandwidth. In most situations a case-specific baseline has to be defined and in the discussion of the consequences of the different new breeding techniques, suggestions are put forward for references that can serve as baseline.

In this report the consequences of four different classes of new plant breeding techniques are discussed. For each class a selection of techniques is discussed in detail because of their potential application in the near future.
Besides the specific technical issues related to the different classes of techniques described below it is clear that general precautions or actions, to prove that a product is free of transgene sequences, have to be undertaken for plants or plant products which are a result of these novel techniques. This can entail to performing PCR tests, protein analyses or any other test capable to attest that transgene sequences, Agrobacterium and chromosomal DNA of Agrobacterium and viral sequences (in case of VIGS related techniques) are not present, in the plants or plant products when the plants or plant products have been exposed to Agrobacterium or virus sequences.

The first class entails different techniques in which genetic modification is used as a tool to facilitate breeding. In these techniques, (partial) genetically modified plant lines are created and these plant lines are subsequently used to create derivatives that are in the end completely free of the genetic material that was used in the initial genetic modification. For this class, four different techniques are described: agroinfiltration, virus induced gene silencing (VIGS), reverse breeding and accelerated breeding following induction of early flowering.
Agroinfiltration is a technique using *Agrobacterium* as a tool to achieve temporarily and local expression of genes that are foreign to the species in a plant. This technique is applied for testing the reaction of target plants to transgenic proteins, or for functional gene analysis in plants. Cuttings or seeds of the selected plants that are *Agrobacterium*-free may be used for further crop development.
VIGS (Virus Induced Gene Silencing) is a technique used for (transient) silencing of the expression of specific endogenous genes in plants. VIGS is mainly used for functional analysis of genes. The VIGS DNA vector is usually introduced into the plant using *Agrobacterium* or specific plant viruses.
Reverse breeding is a novel breeding technique that makes use of genetic modification to facilitate breeding of F1-hybrids by suppression of meiotic recombination. In the final breeding steps the genes used for the genetic modification are crossed out, resulting in end-products that are free of genetic modification-related DNA sequences.
Accelerated breeding is a novel breeding technique that makes use of genetic modification to speed up breeding by induction of early flowering. In the final breeding steps the genes used for the genetic modification are crossed out, resulting in end-products that are free of genetic modification-related DNA sequences.
All these four techniques result in end products that are completely free of any DNA that is related to the genetic modification. Following agroinfiltration and VIGS, plants are recovered

from tissue of the partly genetically modified plant, that was not genetically modified. In reverse breeding and accelerated breeding the foreign DNA sequences are genetically crossed out. The absence of any DNA sequences that are related to the genetic modification also brings about the extreme difficulty if not inability to indicate the end products from this class as being derived from genetically modified progenitors. For all four techniques in this first class it is concluded that in general the consequences for the environment and food and feed safety are not different to those of the baseline. As baseline the original plants that were tested by agroinfiltration or VIGS can be used. In case of reverse breeding and accelerated breeding, plants obtained through conventional breeding are good references.

Plants and products from this first class do not contain any genetic material that was used for the initial genetic modification. Therefore, plants and products obtained through this first class of new plant breeding techniques are similar to the baseline, which are traditionally bred plants, and it follows that the consequences for the environment and food and feed safety do not differ from that of traditionally bred plants. The fact that plants obtained after selection using agroinfiltation or VIGS, or with the help of reverse breeding or accelerated flowering are as safe as traditionally bred plants, justifies the exemption of these plants from the European regulations for genetically modified organisms. General proof that the plants or product is Agrobacterium free, virus free and transgene free should be delivered using accepted standard techniques and/or methods.

A second class entails plants obtained by combining genetically modified and non-genetically modified plants by grafting. From these chimeric plants only the non-genetically modified part is used for harvesting of food, feed or ornamental products. The most obvious grafting involves grafting of a non-genetically modified scion on a genetically modified rootstock. In such a graft, products (like fruits or flowers) are produced on the non-genetically modified plant that is grown on a genetically modified root stock. The combined genetically modified-non genetically modified plants usually have improved cultivation characteristics.
The end products that are harvested from the non-genetically modified scion are completely free of any DNA that is related to the genetic modification of the rootstock. However, depending on the nature of the genetic modification of the rootstock, genetic modification-related RNA molecules, proteins or other metabolites may be transmitted from the rootstock to the scion. Because each genetic modification will have its specific effect on the end products, no general conclusion can be drawn concerning the consequences for the environment and food and feed safety. Therefore for this technique a case-by case evaluation is required to compare the consequences to the baseline. As baseline the same scion grafted on a non-genetically

modified rootstock can serve as a reference. Interspecific grafts may be informative baseline references to display the full bandwidth of consequences of grafting as a technique itself.

Although in this second class, products from the non-genetically modified part of the grafted plants do not contain any genetic material that was used for the initial genetic modification, RNA molecules, proteins and metabolites that are related to the genetic modification may be present. Therefore, plants and products obtained in this class may differ from the baseline, and no general conclusion with respect to consequences for the environment and food and feed safety can be made. General proof that the plants or product is Agrobacterium free, virus free and transgene free should be delivered using accepted standard techniques and/or methods.

A third class of new plant breeding techniques uses genetic modification as a direct tool to introduce new, but in the germplasm occurring, characteristics to a plant. The genetic material used for the modification is originating from the same or a sexual compatible species. Two different approaches, cisgenesis and intragenesis are discussed.

Cisgenesis is the production of genetically modified plants using donor DNA from the species itself or from a cross compatible species. The newly introduced DNA is an unchanged natural genome fragment containing a gene of interest together with its own introns and regulatory sequences, like gene promoter and gene terminator DNA sequences. The introduced DNA is in principle free of vector DNA, however the exception being T-DNA border sequences that are flanking the cisgenic DNA sequences. These short sequences are by nature non-coding and are unlikely to have a phenotypic effect.

Intragenesis is the production of genetically modified plants using donor DNA from the species itself or from a cross compatible species. The difference with cisgenesis is that intragenesis allows the creation of new combinations of DNA fragments which are all originating from the species itself or from a cross compatible species. In intragenesis also the transformation vector itself may be composed of functional DNA fragments from the genome of the target crop species.

Both cisgenesis and intragenesis lead to end products that are containing genetically modified DNA sequences. However, except for the short T-DNA border sequences that are transferred together with the cis- or intragene, the DNA used for the modification is all originating from the species itself or from a cross-compatible species. Although integration of the cisgene will most probably occur in a different position in the genome this does normally not mean that there are inherent differences with regards to level and timing of expression. Thus cisgenesis will lead almost always to phenotypes that also can be achieved by conventional breeding.

Because in intragenesis new combinations of regulatory and coding sequences can be made, the expression of the intragenes is expected not always to correspond to the expression of the native corresponding genes in their natural genomic position. Depending on the nature of the intragenes this may have different consequences for the environment and food and feed safety, when compared to the baseline. If intragenesis is specifically aimed at silencing of a single endogenous gene, the intragenic plants may be comparable to plants with knock-out mutations obtained by mutation breeding. Such plants can be used as baseline. In general, the consequences of intragenesis aimed at gene-silencing of a single gene will be similar to consequences of mutation breeding.

Plants from this third class contain DNA sequences that have been introduced by a genetic modification step. The introduced genes are originating from the species itself or from a cross-compatible species. In case the integration was proven to be outside genes of the recipient genome ánd the introduced genes show an expression that corresponds to the baseline, such cisgenic and intragenic plants are regarded as similar to the baseline, also in terms of environmental safety and food and feed safety. If it is also proven that the final product is free of agrobacteria and sequences that are foreign to the species, this justifies the exemption of such cisgenic and intragenic plants from the European regulations for genetically modified organisms. In general however, intragenesis is aimed at differential expression of genes. If for intragenesis an alternative promoter was used to alter the expression of an intragene, the intragene expression may deviate from that of the baseline. In such a case additional studies are required to assess the environmental and food and feed safety.

The fourth class of new breeding techniques considered in this report concerns techniques where genetic modification is used as a tool to make specific mutations. These techniques introduce site-directed mutations to native genes, leading to a knock-out of gene expression or to changes in the gene-expression pattern or in gene-product properties. One such method involves oligonucleotide-mediated mutation induction.
Oligonucleotide-mediated mutation induction is a promising approach for knocking out or adapting gene function in crops. The method aims at precise and specific mutations of an endogenous gene sequence without the integration of foreign DNA into the plant genome.
So far, oligonucleotide-mediated mutation induction has only been described for mutations that lead to amino acid substitutions into the acetolactate synthase (*als*) gene, resulting in an herbicide-resistant phenotype. Mutants obtained by the described techniques can in principle also be obtained through mutation breeding (using ionizing radiation or chemical mutagens), which has a long history of application in plant breeding and is exempted from EU regulations

for genetically modified organisms. End-products from plants produced by oligonucleotide-mediated mutation induction are therefore similar to plants obtained through mutation breeding, which are a good baseline. The consequences for the environment and food and feed safety are similar to those of the baseline. This may justify the exemption of plants obtained through oligonucleotide-mediated mutation induction from the EU regulations for genetically modified organisms.

Plants and products from this last class of new plant breeding techniques do not contain any genetic material that was used for the initial genetic modification. The genetic modification was used as tool to introduce specific mutations. Plants and products obtained through this class of new plant breeding techniques are similar to the plants obtained by traditional mutation breeding, which are used as baseline references, and it follows that the consequences for the environment and food and feed safety do not differ from that of traditionally mutated plants. The fact that plants from this class are as safe as traditionally bred plants, justifies the exemption of these plants from the European regulations for genetically modified organisms.

Introduction

In the last decennium several new plant breeding techniques have been proposed and developed. These techniques resemble, are derived from, or make indirectly use of genetic modification techniques. The new breeding techniques that are considered in this report share as common feature that in the final products (plants or plant parts) no genes that are foreign to the species are present. Application of these new techniques results in crop phenotypes that could also be obtained through conventional breeding or mutation breeding, but often in a much longer time frame. Under the current conditions these new techniques fall under the European Directive 2001/18/EC. This directive considers all organisms that are generated making use of a genetic modification somewhere during their development as being a genetically modified organism. This brings about that if in such an organism all DNA sequences that are related to the genetic modification are eliminated, this organism, and also its offspring, will be considered a genetically modified organism.

In Europe, the cultivation, trade and use of food and feed of any genetically modified crop is subject to EU regulations and a safety assessment of the genetically modified crop is part of the admission procedure. The assessment for environmental, food and feed safety is a time-consuming and costly (on average €6.8 million for a full assessment (EU)) procedure.

With the advent of new breeding techniques which make the current accepted assessments of determining whether a plant or plant product is a GMO very difficult if not impossible, an update of the European Directive 2001/18/EC is required if these techniques are to be brought into practice. This report describes a number of these new developments or techniques and tries to come up with a technical-scientific approach to assess possible consequences of new breeding techniques for the environment and human & animal health. It furthermore delivers statements with some of these new breeding techniques whether or not they could be exempted from the European Directive 2001/18/EC. Ethical and social aspects of the new techniques are not taken into consideration.

Classical and modern plant breeding

Domestication of crops is a very old process. Breeders continuously improve existing varieties by crossing combinations of crop varieties that lead to more domesticated ones. For breeding improved crops, breeders have in principle the complete gene-pool of a certain species (i.e. all the gene-variation that is present in a species) at their disposal. In order to enlarge the gene pool of a crop, breeders make use of genetic variation that is present in other, closely related species. These are often wild species which may for example be a new source of resistance genes. Many existing crops, like many *Brassica* types and wheat are a result of crossings between different species (inter-specific breeding). The possibility and success of such inter-specific breeding (or

hybridization) may however be limited by natural crossing barriers. More advanced technologies like embryo rescue, polyploidisation and somatic hybridization (cell fusion) may move these barriers further outwards. These technologies make use of skills developed in tissue culture labs and may result in new hybrids that would not have been produced without these techniques. The new hybrids often find their application as parent in breeding programs. Regarding these new techniques, the European Directive 2001/18/EC is only applicable to somatic hybridisation; however, products obtained by somatic hybridisation of crossable species are exempted from this directive. This exemption is motivated by the fact that plants obtained by somatic hybridisation of cross-compatible species in principle can also be obtained using conventional breeding techniques.

Another modern breeding technique is mutation breeding. The occurrence of genetic mutations is a natural biological phenomenon creating new genetic variation. This process of creating new genetic variation can be accelerated by inducing mutations artificially, using ionizing radiation or chemical mutagens producing a high number of genome-wide mutations. Subsequent selection of favorable mutant plants and introducing these in breeding programs has resulted in many food crops that are on the market nowadays. The Food and Agriculture Organization of the United Nations/ International Atomic Energy Agency Mutant Cultivar Database (FAO/IAEA, 2001; Maluszynski, 2001) lists more than 2,200 varieties of various species worldwide that have been developed using induced mutagenesis agents, including ionizing irradiation and ethyl methane sulfonate (see also Ahloowalia et al., 2004). Although crops obtained using mutation breeding techniques are part of the European Directive 2001/18/EC, they are exempted from this directive because of their long history of safe use. Recently, mutation breeding attracted new attention because of the development of a molecular biological technique called ‘tilling’ that allows rapid identification of mutations in genes of interest (Comai and Henikoff, 2006), thereby giving a more focused application of mutation breeding.

Since about twenty years genetic modification has been named and used as a tool with high potential to improve crops. In principle, genetic modification allows the introduction of any new genetic information into the genome of an organism. In practice, genetic modification of plants is not always easy to achieve and success rates differ from species to species. Nevertheless, for many important crops transformation methods have been developed, many genetically improved lines have been produced and several transgenic crops have been commercialized and are grown on a world-wide scale (ISAAA, 2008).

Genetic modification methodology

For genetic modification of plants several methods have been developed. Direct gene transfer (DGT) techniques whereby ‘naked’ DNA is introduced into plant cells were the first to be used. They included amongst others transfer of DNA to protoplasts by using electric currents

(electroporation) or chemicals (Poly Ethylene Glycol) or transfer of DNA to different types of tissue using ballistics. In this latter technique (also called particle bombardment), micro-projectiles coated with DNA are delivered into plants cells. The DNA that is released will integrate into the plant cell genome and subsequent selection of genetically modified cells and regeneration of plants from these cells will result in the ultimate genetically modified plant lines. Of all these DGT techniques only particle bombardment is used extensively and with success. The other DGT techniques were more used for research purposes. Particle bombardment is a robust and relatively efficient method and is especially applied for transformation of monocots like wheat, rice and corn. Using particle bombardment the DNA integration sites and patterns are often complex which makes this technique less favorable for (commercial) plant transformation. The most commonly used genetic modification (or transformation) technique makes use of the natural DNA-vector *Agrobacterium*. Members of this bacterium genus are able to introduce novel DNA into the plant genome. The DNA that is transferred to the plant is part of a so-called DNA plasmid which is present in the *Agrobacterium* cells. This DNA-plasmid can be modified in a molecular (DNA) laboratory and re-introduced in the bacteria using standard molecular biological techniques. Using these techniques optimized plant transformation plasmids (also called binary vectors) have been developed for introduction of genes of interest into the plant genome. The part of the DNA plasmid that is transferred to the plant genome (and contains the gene(s) of interest) is called transfer-DNA (T-DNA) and is delimited by specific 25 base pair long left and right T-DNA border sequences (LB and RB resp.). After integration a partial RB (usually 3 base pairs) and LB (in theory 21-22 base pairs) are usually present in the genome and will define the stably integrated T-DNA in the plant genome. Frequently, the unintentional transfer of additional DNA of the plant transformation plasmid (vector backbone) to the plant genome has been observed. Recently also the occasional transfer of *Agrobacterium* chromosomal DNA has been reported (Ülker et al., 2008).

Since the plant genetic transformation process is a rather inefficient process, selection methods are used to screen for successful transformation events. Most often for this selectable marker genes, such as antibiotic resistance genes and herbicide tolerance genes, are employed and are introduced in the plant genome together with the gene(s) of interest. Although some selectable marker genes, like the kanamycine resistance gene *npt*II, are regarded as safe, marker genes may be undesired in the ultimate genetically modified plant lines. There are several ways to come to genetically modified plants that do not contain selectable marker genes (for overview of methods see: Puchta, 2003). One approach is to eliminate a putative marker by segregating it out by crossing sexually. For crops that are vegetatively propagated or that have a long reproductive cycle (like potato and many fruit crops), sexual crossing is not the method of choice for selectable marker gene removal. For these crops marker elimination methods have

been developed using site-specific recombination. If transformation efficiencies are sufficiently high, one may also perform transformation of plants without the use of selectable marker genes. In such a system, the genetically modified plants will be selected from all the plants that have been regenerated from *Agrobacterium*-mediated transformed plant material using molecular biological techniques (PCR). Recently, in some reports the successful selection of genetically modified plants without the use of selectable marker genes has been demonstrated (de Vetten et al., 2003; Doshi et al., 2007; Jia et al., 2007; Malnoy et al., 2007).

New plant breeding techniques

The new plant breeding techniques considered in this report have a common feature in that they all make use of a genetic modification step (in many cases using *Agrobacterium* as the vector of choice) in the production of the ultimate product. For this reason, all these techniques fall under the European Directive 2001/18/EC. Six different classes of new breeding techniques and accompanying techniques are listed in Table 1. The techniques marked in bold letter type are described in this report and for these techniques the possible consequences to the environment and human & animal health are discussed. These techniques are selected because of their potential application in the near future.

The grafting technique in which a genetically modified scion is grafted on a non-genetically modified rootstock is not considered in this report because application of such a grafting technique has not been described. For the new breeding techniques involving homologous recombination or the application of zinc-finger nucleases (classes 4 & 5 in Table 1) only one example, oligo-induced mutation induction, is described. All these techniques in classes 4 & 5 have exiting potentials, but are still in their infancy and therefore not described in this report. At the final phase of writing this report however, two letters to *Nature* describe the improved applicability of zinc-finger nucleases (Shukla et al., 2009; Townsend et al., 2009), making targeted DNA sequence changes in endogenous plants within reach. These reports also demonstrated the speed at which new plant breeding techniques are currently developing. Hanin and Paszkowskiy (2003) and Puchta (2003b) give a nice overview of techniques using homologous recombination for plant genome modification and Durai et al. (2005) can be consulted for background information regarding the zinc finger nuclease technique. Although epigenetic modification can be seen as an interesting new breeding technique, stable DNA-methylation is difficult to achieve and factors affecting this are poorly understood. Short-term introduction of techniques involving DNA-methylation is not foreseen and therefore this technique is not considered in this report. For general information regarding DNA-methylation the review by Bender (2004) is recommended.

For the techniques that are considered in detail the following aspects will be described and discussed:

-Description of the technique

-Potential application in current breeding programs

-Specific features of application of the technique

-Methods for screening of presence/absence of gene constructs used

-Comparison of end products of new breeding techniques and conventional bred crops

-Environmental consequences

-Consequences for food and feed safety

1. Techniques in which genetic modification is used as a tool to facilitate breeding. In these techniques, (partial) genetically modified plant lines are created and these plant lines are subsequently used to create derivatives that are in the end completely free of the genetic material that was used in the initial genetic modification. a. **Agroinfiltration** b. **Virus Induced Gene Silencing (VIGS)** c. **Reverse breeding** d. **Accelerated breeding following induction of early flowering**
2. Combining genetically modified and non-genetically modified plants by grafting. From these chimeric plants only the non-genetically modified-part is used for harvesting of food or feed products. a. **Grafting of a non-genetically modified scion on a genetically modified rootstock** b. Grafting of a genetically modified scion on a non-genetically modified rootstock
3. Use of genetic modification as a direct tool to introduce new, but to the germplasm belonging characteristics to a plant. The genetic material used for the modification is originating from the same or a sexual compatible species. a. **Cisgenesis** b. **Intragenesis**
4. Techniques where genetic modification is used as a tool to make specific mutations. These techniques introduce site-directed mutations to native genes, leading to a knock-out of gene expression or to changes in the gene-expression pattern or in gene-product properties. a. **Oligo-induced mutation induction** b. Zinc finger nuclease induced mutation (see recent publication in Dutch: Zinkvinger aan de pols Ontwikkelingen en implicaties van de zinkvingertechnologie. COGEM signalering CGM/090616-02) c. Mutation through homologous recombination
5. Techniques using genetic modification to introduce proteins that lead to homologous recombination. a. Zinc finger nuclease induced gene replacement b. Chloroplast transformation (homologous recombination) c. Homologous recombination
6. Epigenetic modification. a. Gene-inactivation through DNA-methylation

Table 1. *Different classes of new breeding techniques and accompanying techniques are listed in this table. The techniques marked in bold letter type are selected because of their potential application in the near future and are discussed in this report.*

Baseline

To be able to discuss the consequences of new breeding techniques for the environment and for food and feed safety, a (case-specific) baseline has to be determined. The baseline is described using references that cover the 'natural' situation in its full bandwidth. The most obvious reference is the (conventionally bred) genotype that was used for the genetic modification, but screening of additional genotypes is relevant to indicate the bandwidth for each parameter. Moreover, products in other species with a history of safe use can also serve as important references. If for example in a genetically modified-fruit a specific compound is present in abundance exceeding the species specific baseline, this may be just a natural level in fruits from a different species. Next to this, mutants, somatic hybrids of crossable species and grafted plants may be useful references to determine the bandwidth of the baseline. It should be stressed however, that certain references, for example a putative specific mutant plant, will not always be available, although it is evident that such a reference can be produced according to conventional plant breeding techniques.

Agroinfiltration

Agroinfiltration is a technique using *Agrobacterium* as a tool to achieve temporarily and local expression of genes that are foreign to the species in a plant. This technique is applied for testing the reaction of target plants to transgenic proteins, or for functional gene analysis in plants. Cuttings or seeds of the selected plants that are *Agrobacterium*-free may be used for further crop development.

Agrobacterium tumefaciens is commonly used for the stable genetic modification of plants. However, *Agrobacterium* can also be used to achieve transient gene expression in plants. During transient gene expression the genes that are introduced in the host cells are not necessarily incorporated into the plant genome, but rather become temporarily active as free DNA molecules in the plant cell. This results in a rapid transcription into RNA molecules (messenger RNA (mRNA), in case of genes which are expressed into proteins, or double-stranded RNA (dsRNA) when so-called RNAi constructs are used to block endogenous gene expression). Transient gene expression is obtained by infiltrating *Agrobacterium* cells into plant parts, usually leaves or stem tissue, by using an infiltration with a needleless syringe (see Fig. 1). This technique is called agroinfiltration and a few days after agroinfiltration the plants can be screened for the intended effects.

Agroinfiltration is mainly applied as a diagnostic disease resistance test in plants.

Initially, agroinfiltration has been described as a method for effective virus inoculation of plants. In that case we rather talk about agroinoculation or agroinfection. Because mechanical virus inoculations often have a lack of success, agroinoculation was developed using *Agrobacterium* as a delivery agent for viral genomes. For this, the viral genome was isolated from the virus to be tested and was subsequently cloned between the left and the right border of the T-DNA of a plant transformation vector. To assess the plant's susceptibility to virus infection, *Agrobacterium* equipped with this vector is infiltrated into the plant by agroinoculation. Consequences of agroinoculation techniques are similar to those of virus-induced gene silencing (VIGS) and will be described in the section *'Virus-induced gene silencing'*.

Agroinfiltration is often used as a diagnostic test by transiently expressing genes coding for avirulence proteins into the plants to be tested. These genes originate from plant pathogens and may interact with plant resistance genes leading to a defence response in the plant. The reaction of the plant to these avirulence proteins may indicate a certain level of resistance or tolerance. Plants with a desirable resistance pattern will then be selected and propagated for further evaluation and/or breeding.

Agroinfiltration is also used as a method to transiently express gene constructs for the induction of gene silencing (or down regulation of gene expression) in plants. One such method involves the introduction of DNA constructs containing inverted repeats for the production of dsRNAs into the plant. These constructs effectively induce RNA silencing by RNA interference (RNAi) leading to degradation of target mRNAs. RNAi has as direct result the down-regulation of the expression of the corresponding gene. The way RNAi works has been elucidated almost completely and one of the lesser understood aspects of RNAi is that it also can induce RNA-directed DNA methylation (Mathieu and Bender, 2004). This DNA methylation (directed towards the gene to which the RNAi sequence is directed) leads to alteration at the DNA or chromatin level. Methylation of promoter sequences may result in prevention of transcription (= production of mRNA) of the target genes (Mette et al., 2000), whereas DNA methylation of transcribed regions usually has no effect on gene expression. Nevertheless, some reports describe that methylation of transcribed regions can lead to both silencing (Hohn et al., 1996) and up regulation (Li et al., 2008; Shibuya et al., 2009) of gene expression.
Agroinfiltration is also used for the introduction of virus vectors for VIGS. Consequences of the application of VIGS will be discussed in the section '*Virus-induced gene silencing'*.

Figure 1. *Infiltration of a suspension of Agrobacterium cells into a tobacco leaf.*

Potential application in current breeding programs

Agroinfiltration is mainly a tool of interest in breeding programs to test for resistance against viral, bacterial or fungal disease. After testing, depending on the crop species used, seeds or vegetative tissue will be harvested from the selected plants and used for further breeding or

multiplication. So far, the application of agroinfiltration is restricted to help answering research questions only.

Specific features of application of the technique

It has been shown that after agroinfiltration *Agrobacterium* is able to persist at the site of inoculation for a long time (Moligner et al., 1993). To prevent secondary infections with *Agrobacterium* in further crop development, seed or other tissue will be harvested from locations other than the site of agroinfiltration. However, it has been found that *Agrobacterium* is able to move internally through the xylem vessels in grape (Tarbah et al., 1987). Recently it has also been described that natural pathogenic agrobacteria were able to move systemically inside the plant leading to the induction of secondary infection sites beyond the site of inoculation for a number of plant species (tomato, rose, grapevine, chrysanthemum, cherry, a peach x almond hybrid and in walnut) (Cubero et al., 2006). So, a consequence of agroinfiltration may be that *Agrobacterium* moves from the site of infiltration throughout the whole plant to the parts used for further propagation, causing infection and possibly stable transformation (i.e. stable DNA integration into the plant genome).

Although, several genera of bacteria have been found within the seeds of plant species (Schaad et al; 1982), *Agrobacterium* ssp. are generally thought not to be seed transmitted. However, using TaqMan PCR, an extreme sensitive screening technique, with an *Agrobacterium* ssp.-specific TaqMan probe, Weller et al. (2002) were able to detect (wildtype) *Agrobacterium* in one out of approximately 7.000 surface-sterilized (non-genetically modified) *Brassica napus* seeds, indicating that survival of *Agrobacterium* inside seeds is probably a rare event, but cannot be excluded. This also implies that it cannot be excluded that the DNA introduced in the plant tissue can be incorporated into the nuclear DNA, although no reports are known about this at present time. The positive PCR for the single seed could also be due to the presence of dead *Agrobacterium* cells that were still attached to the seed surface or to PCR errors. From limited information on the fate of *Agrobacterium* in seeds of agroinfiltrated plants it is not possible to conclude whether or not *Agrobacterium* is able to infect seeds upon agroinfiltration. Although it is clear that the probability of internal infections of seeds is very limited, any plant part taken from agroinfiltrated plants with the aim of further propagation should be carefully screened for absence of the *Agrobacterium,* chromosomal *Agrobacterium* DNA and the T-DNA used for the agroinfiltration.

Methods for screening for absence of Agrobacterium and transgenes

In principle the plants that are subjected to an agroinfiltration test can be cloned before testing, in order to prevent the unintended presence of agrobacteria in any follow-up plant material.

However, in breeding programs usually large numbers of progeny have to be screened, which makes clonal propagation of all progeny economically impractical.
To avoid latent infections of *Agrobacterium* and to avoid the presence of stable transformed cells (so containing T-DNA and/or bacterial DNA) in the ultimately selected plant material, effective and sensitive detection methods such as PCR or quantitative PCR (qPCR) (Cubero et al., 1999; Sudarshana et al., 2006) are used. Using qPCR, Sudarshana et al. (2006) were able to detect as little as 20 *Agrobacterium* cells per gram of soil. Cubero et al. (2006) used a methodology based on a combination of (bacterial) isolation methods and PCR technology for detecting agrobacteria in plant tissue. From their results it can be concluded that methods based on enrichment of agrobacteria using selective culture media (plant extracts are added to optimized and selective culture medium) in combination with PCR are more effective than applying PCR directly on DNA extracted from plant material.

Comparison of end products of new breeding techniques and conventionally bred crops
If the end products of agroinfiltrated plants are derived from non-genetically modified plant parts (e.g. meristems or seeds) that have been proven to be completely free of *Agrobacterium, Agrobacterium* genomic DNA sequences or integrated T-DNA, the end product will be exactly similar to the original plants used for agroinfiltration. After agroinfiltration the presence and expression of the introduced genes is transient and the gene effect will fade away in time.
In case agroinfiltration is used for the induction of RNA silencing, the silenced phenotype can be maintained through vegetative propagation or organ regeneration and can even be transmitted through a graft (Sonoda et al., 2000; Tournier et al., 2006). Transmission of the RNA-silencing signal through seed has not been reported. Davuluri et al. (2005) found that when crossing a transgenic tomato plant with a RNA-silenced phenotype with a non-transgenic tomato, the progeny that had not received the transgene also did not show the silenced phenotype, indicating that the silencing signal was not transmitted through seeds.
If agroinfiltration is aimed at induction of RNAi-directed DNA methylation of promoter regions (promoter silencing), the methylation-related phenotype occasionally can be stably inherited by the next sexual generation, independent of the presence of the transgenes (Park et al., 1996). So, although the resulting offspring can be regarded as non-genetically modified, the result of the genetic modification can still be effective in following sexual generations.

Environmental consequences
The main risk of application of agroinfiltration is the unintended release of genetically engineered *Agrobacterium* strains into the environment. In the soil, *Agrobacterium* is able to survive and may transfer transgenes to other plants. Next to this, binary vectors may be transferred to other microorganisms via horizontal gene transfer (Droege et al., 1999; Stewart et

al., 2000). To prevent the release of *Agrobacterium* or the spread of recombinant DNA, reliable and validated methods have to be applied to prove that planting material, including seeds, originating from agroinfiltrated plants is completely free of *Agrobacterium* and binary vector sequences. If so, this *Agrobacterium*- and recombinant DNA-free plant material is similar to the original plants before agroinfiltration. These plants can serve as a baseline. In principle, no environmental consequences are foreseen when *Agrobacterium*-free plant material originating from agroinfiltrated plants is released into the environment. In case agroinfiltration was used for gene-silencing, the silencing effect occasionally may still be present in the non-genetically modified vegetative or sexual offspring of the agroinfiltrated plant. Therefore the expression of the genes that have been silenced should be carefully evaluated and compared to the baseline. If they do not deviate from the baseline or from the bandwidth of expression than there would be no reason to expect an effect on the environment.

Consequences for food and feed safety

Crop plants that are originating from parental plants which are selected using agroinfiltration should be proven to be free of *Agrobacterium, Agrobacterium* chromosomal DNA and binary vector sequences before they are released into the environment. Food products harvested from these crop plants are therefore also free of *Agrobacterium, Agrobacterium* chromosomal DNA and recombinant DNA sequences. The food and feed safety will be similar to that of products harvested from the original plants before agroinfiltration or from corresponding plant lines that are produced in a similar way, but without the help of genetic modification. These plants can serve as a baseline.

If agroinfiltration was aimed at gene silencing, the silencing effect occasionally may still be present in the non-genetically modified vegetative or sexual offspring of the agroinfiltrated plant. This may result in changes in expression of the target genes that have been silenced. In such a case the offspring should be carefully checked for possible changes in target gene expression and again when similar to the baseline levels or falling within the bandwidth of expression no food or feed safety issues are expected.

Virus-induced gene silencing

Virus-induced gene silencing (VIGS) is a technique used for (transient) silencing of the expression of specific endogenous genes in plants. VIGS is mainly used for functional analysis of genes. The VIGS DNA vector is usually introduced into the plant using *Agrobacterium* or specific plant viruses.

Application of virus-induced gene silencing (VIGS) is a powerful technique for silencing the expression of specific genes transiently in a host plant through RNA interference (RNAi) (Baulcombe, 1999; Ratcliff et al., 2001). VIGS can give similar gene silencing results as described in the section '*Agroinfiltration'*, but for VIGS double-stranded RNA (dsRNA) is produced by viral replication of viral single stranded RNA, rather than by transcription of introduced genes (DNA). Like with agroinfiltration, VIGS avoids the need for time-consuming plant transformation and regeneration processes. VIGS is based on a plant defense mechanism that limits the severity of natural virus infection in plants (Baulcombe, 1999). VIGS can be induced under laboratory/greenhouse conditions by introducing VIGS vectors into plants. These viral vectors are composed of a modified viral genome and include a fragment from the host plant gene to be silenced. Several methods are commonly employed to deliver VIGS vectors to plants, like mechanical inoculation using (in vitro transcribed) viral RNA or extracts from infected leaves (containing a VIGS-specific virus) and agroinoculation (using *Agrobacterium).* Inoculation results in replication of the virus and production of dsRNA intermediates. These intermediates are recognized by the plant cell as foreign products which results in activation of the plant defense mechanism. This subsequently leads to degradation of the dsRNA into small RNA molecules called short interfering RNAs (siRNAs). Finally, these siRNAs serve as guides in an RNA-induced silencing complex, leading to specific degradation of messenger RNAs (mRNAs) with identical homology (Chicas and Macino, 2001). So, by the introduction of RNA-molecules that are homologous to a specific native gene, the expression of this gene will be silenced through degradation of its corresponding mRNA.

Potential application in current breeding programs

So far, VIGS is mainly used as a rapid test for functionality of specific native genes by silencing its expression. This may for example be applicable to genes that are part of a disease resistance mechanism. Plants showing the expected reaction will be selected for further evaluation (Robertson, 2004). VIGS may also be used as new breeding technique for silencing certain genes, with the aim to achieve an altered plant phenotype which is beneficial for further breeding or propagation. For example silencing of floral repressor genes may induce early flowering (Michaels and Amasino; 1999, 2001) which is useful in speeding up breeding of

species with a long generation time. At this moment the application of agroinoculation and VIGS is restricted to help answering research questions only.

Specific features of application of the technique

For VIGS several different DNA and RNA viruses are modified to serve as vector for gene silencing. Inoculation of plants (irrespective of the method used) with VIGS vectors leads to viral replication and movement of the viruses throughout the plants (Voinnet et al., 2000). For the recovery of virus-free progeny of VIGS-selected plants by clonal propagation, specialized and laborious techniques like thermotherapy and meristem-tip culture are needed (Walkey, 1981). This makes clonal propagation of VIGS-selected plants unsuitable as straightforward method for obtaining virus-free plant material for further evaluation. Depending on the type of virus used and plant species used, seeds may be suitable for further propagation of virus-free VIGS-selected lines. However, some viruses used for VIGS, like the barley stripe mosaic virus (BSMV), are seed transmittable (Johansen et al., 1994). Because each type of virus has, in combination with the plant species used, its own properties, a case-specific evaluation is required to determine if and how virus-free plant material can be produced for propagation of VIGS-tested plants.

In contrast to animal and bacterial viruses, there are no reports of plant virus sequences that have been integrated into the host plant genome after viral infections. Nevertheless, it appears that all members of the plant kingdom have integrated remnant sequences of certain DNA viruses, indicating that occasionally viruses have been integrating into plant genomes throughout evolution (Hull et al., 2000). For plant viruses having RNA genomes there are no such examples of integrated forms (Harper et al., 2002).

If agroinoculation is used to deliver VIGS sequences into the plant, these sequences may be stably integrated into the host plant genome as a result of T-DNA-mediated integration. This is because for agroinoculation the VIGS DNA sequences are first cloned into the T-DNA of a plant transformation vector (binary vector) which subsequently is transferred to *Agrobacterium*. After infiltration of *Agrobacterium* into the plant, the T-DNA containing the VIGS DNA sequences will integrate frequently into the plant genome like as it happens in 'normal' *Agrobacterium*-mediated plant transformations. As an additional consequence of agroinoculation, *Agrobacterium* cells may spread systemically throughout the infiltrated plant. This consequence has been described in detail in the section describing the new plant breeding technique '*Agroinfiltration*'.

Methods for screening of presence/absence of VIGS sequences and corresponding viruses

For VIGS defined DNA sequences are introduced into plants. The presence of VIGS sequences and the related viruses can therefore reliably be determined using PCR techniques (see e.g.

Schneider et al., 2004; Bruun-Rasmussen et al., 2007). Also Southern blot analysis has been used to detect VIGS-derived sequences (Johansen et al., 1994). In case of the use of agroinoculation, PCR tests for the detection of *Agrobacterium tumefaciens* chromosomal DNA and T-DNA need to be performed as well.

Comparison of end products of new breeding techniques and conventional bred crops

After application of VIGS, seeds or vegetative tissue can be harvested from selected VIGS-tested plants and propagated for further breeding or multiplication. If these propagated plants are completely free of recombinant virus or VIGS vector-DNA (and of *Agrobacterium* and *Agrobacterium* DNA when agroinoculation is used), then the end products are in principle similar to the original plants before VIGS. However, despite the absence of VIGS-related DNA or virus, the RNA-silencing signal can persist and the silenced phenotype can be maintained through vegetative propagation (Vaistij et al., 2002). Transmission of the RNA-silencing signal through seed has not been reported.

If VIGS has resulted in the induction of RNA-directed DNA methylation, the methylation-related phenotype occasionally can be stably inherited by the next sexual generation, independent of the presence of VIGS-related DNA or virus (Park et al., 1996). So, although the resulting offspring can be regarded as non-genetically modified (there is no difference at the genomic-DNA sequence level), the result of the genetic modification can still be effective in following sexual generations.

Environmental consequences

The main risk of application of VIGS is the unintended release of the recombinant virus into the environment. Because RNA viruses are used in their entirety for VIGS vectors, unintentional inoculation by mechanical transmission must be considered as a serious consequence. Viruses from DNA vectors lack a protein coat and are not infectious. However, it was shown that for example geminiviruses can evolve rapidly under field conditions and are able to recombine with other geminivirus strains that are present in the same plant (Pita et al., 2001).

The most realistic way to recover VIGS- and virus-free plants is through seeds when non-seed-transmittable viruses have been used for VIGS. If planting material originating from VIGS-tested plants is proven to be completely free of VIGS DNA sequences or VIGS-related viruses, this material is in principle similar to the original plant before application of VIGS and therefore no environmental consequences are foreseen when releasing such material in the environment. As baseline the original plants used for VIGS or similar plants obtained by screening without the help of VIGS can be used. Because VIGS is generally used for gene silencing, the silencing effect occasionally may still be present in the non-genetically modified vegetative or sexual offspring of the VIGS-tested plant. Therefore the expression of the genes

that have been silenced should be carefully evaluated and compared to the baseline. If the expression of the target gene is normal (or rather falls within the bandwidth measured or reported for the original plant) compared to the original plant then there should be no effect on the environment.

Consequences for food and feed safety

Since all plant material will be free of VIGS vector sequences or VIGS-related viruses, the food and feed safety will in principle be similar to the non-genetically modified corresponding food. As baseline similar plants obtained by screening without the help of VIGS can be used. Because VIGS is generally used for gene silencing, the silencing effect occasionally may still be present in the non-genetically modified vegetative or sexual offspring of the VIGS-tested plant. This may result in changes in expression of the target genes that have been silenced. In such a case the offspring should carefully be checked for possible changes in target gene expression. In case expression is similar as in the original plant the food and feed safety is expected to be similar to that of the original plant.

Reverse breeding

Reverse breeding is a novel breeding technique that makes use of genetic modification to facilitate breeding of F1-hybrids by suppression of meiotic recombination. In the final breeding steps the genes used for the genetic modification are crossed out, resulting in end-products that are completely free of genetic modification-related DNA sequences.

Traditionally, varieties of many crops are produced as F1-hybrids. Elite F1 hybrids are developed by an initial careful selection of homozygous parental lines followed by generation of experimental hybrids which are tested for their agricultural or horticultural value. In the initial parental line selection two different parent varieties are inbred (backcrossed repeatedly) for a number of generations to the extent that they are almost homozygous. The divergence between the parental lines promotes improved growth and yield characteristics of the resulting heterozygous F1-hybrid through the phenomenon of heterosis or hybrid vigour. The homozygosity of the parental lines ensures a phenotypically uniform heterozygous F1 generation. The heterozygosity of the F1-hybrids will result in loss of its elite characteristics when the F1-hybrids themselves are used for breeding.
Reverse breeding is a novel method that allows breeders to produce a new hybrid in a much shorter time than with conventional techniques (van Dun et al., 2005; Dirks et al., 2006). Reverse breeding starts with an elite heterozygous line and aims at the generation of homozygous parental lines. Subsequent hybridisation of these homozygous parental lines produces F1 hybrid plants in which the original genetic composition of the elite heterozygous line is reconstituted. To achieve the homozygous parental lines a complex procedure is followed. First, meiotic recombination is suppressed in the elite heterozygous line. For this the heterozygous line is genetically modified by the introduction of gene silencing constructs to down-regulate the expression of genes, like *dmc1* and *spo11*, which are involved in the meiotic recombination process. From flowers from the resulting transgenic elite heterozygous line, haploid microspores (immature pollen grain) are harvested and the genome of these haploid microspores will subsequently be doubled using a laboratory technique called doubled haploid technology. Using tissue culture techniques the diploid (double haploid) microspores will be developed into embryos and subsequently in homozygous diploid plants. Among a collection of these homozygous diploid plants, parental pairs will be selected that together reconstitute the genetic composition of the original elite heterozygous elite line. Selection of homozygous parental plants that do not contain transgenes ensures that the resulting F1-hybrids are non-genetically modified. This novel plant breeding approach offers clear advantages over existing techniques due to the fact that in principle any heterozygous plant can now be commercially exploited through re-synthesis of suitable parental lines.

Potential application in current breeding programs

Reverse breeding is still in its research phase, but is clearly a technique with high potential. The main advantage of reverse breeding is that using reverse breeding, elite heterozygous plants can be produced as F1 hybrids because of re-synthesis of parental lines.

Specific features of application of the technique

Reverse breeding is a novel breeding technique that makes use of genetic modification in an early step in the breeding process. In successive steps non-genetically modified parental lines are selected to continue breeding with, guaranteeing that the F1-hybrid offspring will be completely free of DNA sequences that are related to the genetic modification.

Methods for screening of presence/absence of gene constructs used

One critical step in the process is the reliable selection of parental plant lines that are completely free of genetic modification-related DNA sequences. Standard PCR techniques are suitable to reliably confirm the presence or absence of transgenes into the selected lines for further breeding. Next to this, Southern blotting using the complete T-DNA construct used for the genetic modification as probe, gives additional evidence for the genetic modification-free status of the selected parental lines.

Comparison of end products of new breeding techniques and conventional bred crops

The parental lines produced by reverse breeding can in principle be obtained in a similar way using conventional breeding techniques, but in a much longer time-frame. The end-products of the reverse breeding will therefore be similar to parental lines obtained by conventional breeding.

The initial genetic modification step involves gene silencing using RNA interference (RNAi). It is known that RNAi occasionally can lead to RNA-directed DNA methylation of the transcribed region, which can give a change in expression of the target genes and therefore in a changed phenotype. The RNA silencing signal itself will not be transmitted through seeds, but a methylation-related changed phenotype can be stably inherited by the next sexual generation (Hohn et al., 1996; Park et al., 1996). So, although the resulting offspring can be regarded as non-genetically modified, the result of the RNAi can still be effective in following sexual generations. However, in the specific case of reverse breeding, a possible RNA-directed DNA methylation of the genes involved in the meiotic recombination process is expected not to have an effect on the end products. The parental plants and F1 hybrids will have no changed phenotype because of changes in meiotic recombination. Moreover, variation in meiotic recombination is a natural occurring phenomenon (Wijnker and de Jong, 2008).

Environmental consequences

Because the parental lines produced by reverse breeding and the subsequent produced F1-hybrids do not contain any genetic modification-related DNA sequences and because a possible RNA-directed DNA methylation that is transmitted to the offspring will only have an effect on meiotic recombination, the consequences for the environment are in principle similar to those of parental lines and F1-hybrids obtained by conventional breeding. Such conventionally bred parental lines and F1-hybrids can serve as baseline.

Consequences for food and feed safety

The F1 hybrids are usually used for food and feed production. Therefore, the F1-hybrids are considered for the consequences for food and feed safety, rather than the parental lines, which are the actual end products of reverse breeding. F1-hybrids obtained by crossing of reverse breeding-derived parental lines do not contain any genetic modification-related DNA sequences and a possible RNA-directed DNA methylation that is transmitted to the offspring will only have an effect on meiotic recombination. Therefore, products from these hybrids are as safe as products obtained from the original heterozygous line used for reverse breeding or as conventionally bred F1-hybrids. The original heterozygous line or conventionally bred F1-hybrids can serve as baseline.

Accelerated breeding following induction of early flowering

Accelerated breeding is a novel breeding technique that makes use of genetic modification to speed up breeding by induction of early flowering. In the final breeding steps the genes used for the genetic modification are crossed out, resulting in end-products that are completely free of genetic modification-related DNA sequences.

In comparison to herbaceous plants, the breeding of trees is more time-consuming due to their long generation time. Shortened juvenility and precocious flowering are therefore important breeding goals. Flower initiation has been intensively studied in *Arabidopsis* and orthologues/ homologues of genes involved (*LEAFY* (*LFY*), *APETELA1* (*AP1*), and *TERMINAL FLOWER* (*TFL1*)) have been cloned from amongst others apple (Wada et al., 2002; Kotoda and Wada, 2005; Flachowsky et al., 2006). One approach to shortening the juvenile phase of perennial crops is to reduce juvenility/vegetative maintenance factors, such as *TFL1*, by gene silencing. Gene silencing of genes like *TFL1* can results in early flowering leading to a drastically reduction of the time of breeding cycles (Flachowsky et al., 2006). Next to this, overexpression of BpMADS4, a flower initiation related transcription factor gene from silver birch (Elo et al., 2001) has shown to induce early flowering in apple (Flachowsky et al., 2007).
Using gene silencing or overexpression constructs, genetically modified plants can be produced that flower much earlier than the original non-genetically modified lines. These early flowering genetically modified plants will successively be used in breeding programs until the required level of breeding has been reached. In a final breeding step, the transgenes used for induction of early flowering will be crossed out and plant lines that are completely free of genetic modification-related DNA sequences will be selected.

Potential application in current breeding programs
Accelerated breeding using early flowering genes is still in its research phase. Initial experiments have shown that in apple the generation time can be reduced from 5-7 years to just one year (Flachowsky et al., 2009).

Specific features of application of the technique
Accelerated breeding is a novel breeding technique that makes use of genetic modification in an early step in the breeding process. In the final steps non-genetically modified lines are selected to finish breeding with, guaranteeing that the final offspring will be completely free of genetic modification-related DNA sequences.

Methods for screening of presence/absence of gene constructs used

One critical step in the process is the reliable selection of parental plant lines that are completely free of genetic modification-related DNA sequences. Standard PCR techniques are suitable to reliably confirm the presence or absence of transgenes into the selected lines for further breeding. Next to this, Southern blotting using the complete T-DNA construct used for the genetic modification as probe, gives additional evidence for the genetic modification-free status of the selected parental lines.

Comparison of end products of new breeding techniques and conventional bred crops

Elite varieties produced by accelerated breeding can be obtained in a similar way using conventional breeding techniques, but in a much longer run. The end-products will therefore be similar to their counterparts produced by conventional breeding. It is known that RNAi occasionally can lead to RNA-directed DNA methylation of the transcribed region, which can give a change in expression of the target genes and therefore in a changed phenotype. The RNA silencing signal itself will not be transmitted through seeds, but occasionally a methylation-related changed phenotype can be stably inherited by the next sexual generation (Hohn et al., 1996; Park et al., 1996). So, although the resulting offspring can be regarded as non-genetically modified, occasionally the result of the RNAi can still be effective in following sexual generations. In the specific case of induction of early flowering, a possible RNA-directed DNA methylation of the genes involved in the flowering process will result in a clear phenotype (early flowering). Although early flowering can be easily selected against, an early flowering phenotype is expected not to have an effect on the end products.

Environmental consequences

Because new varieties produced by accelerated breeding do not contain any genetic modification-related DNA sequences, the consequences for the environment are similar to those of the conventionally bred varieties. Such plants produced by conventional breeding can serve as baseline. In case RNAi-mediated gene silencing has been used to induce early flowering, there is a possibility that this still induces early flowering in the final sexual offspring (which is free of genetic modification-related DNA sequences). No environmental consequences are expected of an early flowering phenotype. Of course the resulting plants should be checked for the absence of recombinant or GM related DNA by molecular techniques such as PCR or Southern blotting. If that is the case there are no environmental risks.

Consequences for food and feed safety

Because new varieties produced by accelerated breeding do not contain any genetic modification-related DNA sequences, products from these hybrids are as safe as products obtained from conventionally bred varieties. Such plants produced by conventional breeding

can serve as baseline. In case RNAi-mediated gene silencing has been used to induce early flowering, there is a possibility that this still induces early flowering in the final sexual offspring (which is free of genetic modification-related DNA sequences). No consequences are expected of an early flowering phenotype.

Combining of genetically modified and non-genetically modified plant parts by grafting

By combining genetically modified and non-genetically modified plant parts, products (like fruits or flowers) can be produced on non-genetically modified plant parts that are grafted on genetically modified root stocks. The combined genetically modified-non genetically modified plants usually have improved cultivation characteristics.

Grafting is a method of plant propagation where usually stems or buds of one plant are fused with a rooted stem of another. It is most commonly used for the commercial cultivation of fruit trees, grapes, tomatoes, cucumbers and some flowers like roses. For grafting, one plant is selected for its roots and is called the rootstock. The other plant is selected for its stems, leaves, flowers or fruits and is called the scion. Successful grafting requires that a vascular connection between the two tissues is established. This connection allows vascular transport between rootstock and scion.

Grafting a non-genetically modified scion on a genetically modified rootstock results into a chimeric plant from which products can be harvested of the non-genetically modified scion part, so these products are not genetically modified. However, according to the current regulations, if part of the plant is genetically modified, the entire plant should be considered as being genetically modified. There are a number of ways in which genetically modified-rootstocks can be useful in grafting. Using genetic modification, the characteristics of a rootstock, like rooting ability on heavy soils or resistance to soil-born diseases, can be improved. Such an improvement of the rootstock will eventually lead to a better performance of the scion.

Another potential application of genetically modified rootstocks is using it as source of gene silencing through RNA interference (RNAi) (Kalantidis, 2004). RNAi is a natural defense mechanism that causes sequence-specific RNA degradation of invading foreign DNA or RNA molecules (like those from viruses) and can also be used to silence the expression of specific endogenous genes. It has been demonstrated that the RNAi silencing signal in plants is mobile and can spread through the entire plant (Palauqui et al., 1997). In grafted plants, the silencing signal can also transmit through the graft (Sonoda et al., 2000; Crété et al., 2001; Shaharuddin et al., 2006; Tournier et al., 2006). RNAi-rootstocks may therefore be used to silence the expression of specific genes in non-genetically modified scions.

This section is limited to the situation in which a non-genetically modified scion is grafted on a genetically modified rootstock. The reverse situation, in which the scion is genetically modified, is not considered because application of such a grafting technique has not been reported.

Potential application in current breeding programs

Grafting is commonly used for the commercial cultivation of fruit trees, grapes and ornamentals like roses. So far, the use of genetically modified rootstocks has not been reported for commercial production.

Specific features of application of the technique

Stegemann and Bock (2009) reported recently about the transfer of plastid genetic information in a graft from rootstock cells to the cells of the scion and visa versa. Chimeric cells were recovered from the graft site by application of a strong selection pressure. It was not clear whether the genetic information was transferred as DNA fragments or as entire plastid genome or as plastid. Because the genetic exchange was restricted to the graft sites only, products (like flowers and fruits) harvested from a non-genetically modified scion that is grafted on a genetically modified rootstock will not contain genetically engineered DNA sequences from the rootstock and are therefore not genetically modified. The non-genetically modified scion may however contain metabolites, proteins and (small) RNA molecules that are transported from the genetically modified rootstock to the scion and are related to the genetic modification. The consequences of the presence of such genetically modified-related transported products depend on their nature and also on the abundance of the product. The following situations occur after grafting a non-genetically modified scion on a genetically modified rootstock.

a. no transmission of products

If grafting does not lead to transmission of genetically modified-related products to the scion, the genetically modified rootstock will have no direct consequences for the scion.

b. transmission of RNAi for silencing endogenous genes

Under certain conditions, the use of a genetically modified rootstock containing an RNAi construct for gene silencing will result in mobilization of the RNAi silencing signal to the scion. The silencing effect on the scion and its products depends completely on the target of the RNAi construct. Possible target genes may be involved in modifying quality characteristics having a direct effect on the primary products to be harvested, e.g. fruits, but also a secondary effect after clonal propagation or regrafting. It has been found by Sonada et al. (2000) that when a non-genetically modified scion, that was silenced as a result of grafting on a silencing (RNAi) rootstock, was regrafted onto a non-genetically modified rootstock, the silenced phenotype was maintained and the silencing was even transmitted to the new non-genetically modified rootstock. This means that RNAi-silencing can result in a stably altered (non-genetically modified) scion phenotype and products harvested from the non-genetically modified scion may have an altered phenotype that is still related to the original genetic modification.

RNAi-rootstocks can also be used to facilitate breeding, for example by inducing early flowering caused by RNAi-mediated silencing of floral repressor genes (Flachowsky et al., 2007) or for repression of meiotic recombination as used in reverse breeding (Dirks et al., 2006). It was demonstrated by Sonada et al. (2000) that after sexual crossing, the progeny of the non-genetically modified scion, which was grafted on a genetically modified (silencing) rootstock, did not show silencing anymore (Sonada et al., 2000). This means that the RNAi signal is not transmitted to the progeny and that the source of the RNAi signal is not integrated into the scion genome. Therefore, if RNAi-silencing is used to assist breeding, the offspring can be regarded as non-genetically modified because they are both genetically and phenotypically similar to their non-genetically modified counterparts.

As a side-effect however, RNAi silencing occasionally can induce RNA-directed DNA methylation (Mathieu and Bender, 2004). DNA methylation leads to alteration at the DNA or chromatin level and methylation of transcribed regions can lead to both silencing (Hohn et al., 1996) and upregulation (Li et al., 2008; Shibuya et al., 2009) of gene expression. The methylation-related phenotype occasionally can be stably inherited by the next sexual generation, independent of the presence of the transgenes (Park et al., 1996). So, although the resulting offspring can be regarded as non-genetically modified, the result of the genetic modification can still be effective in following sexual generations.

c. transmission of proteins and metabolites

Besides nutrients, the phloem sap contains mRNAs, proteins and metabolites. Hoffmann-Benning et al. (2002) identified and characterized a large number of small proteins in phloem exudate, many of which occur at low concentration. Like metabolites, phloem-specific proteins are translocated into the phloem and have been demonstrated to be graft transmittable (Golecki et al., 1998, 1999). Dutt et al. (2007) proved the presence of transgenic protein in non-transgenic grapevine that was grafted on a rootstock producing transgenic antimicrobial protein. Dependent of the nature of the gene(s) that are used for the modification of the genetically modified-rootstock, the consequence of transmission of the genetic modification-related metabolites and proteins can be very different. For example, metabolites like auxins and cytokinins have a long-distance signaling-function and may have a wide downstream effect on the physiology of the scion. Also proteins like transcription factors or other protein factors involved in gene regulation may be transported and exert their action at long distance. For example a recently discovered flower transition signal appeared to be a protein factor that is transported from leaves to the shoot apex (Corbesier et al., 2007).

Altogether, it is clear that the consequences of grafting a non-genetically modified scion on a genetically modified rootstock can be rather divers and a case-by-case evaluation is necessary to evaluate these consequences.

Methods for screening of presence/absence of gene constructs used

Standard PCR techniques can be used to confirm presence of transgenes into the rootstock. Because the scion is non-genetically modified, there is no need to screen products harvested from the scion for presence or absence of transgenes.

Comparison of end products of new breeding techniques and conventional bred crops

If non-genetically modified scions are grafted on genetically modified-rootstocks, products harvested from the non-genetically modified scion can have an altered phenotype that is related to the genetic modification. The consequence of the altered phenotype is dependent on the nature of the genetic modification of the rootstock.

In case the effects of the modification are completely restricted to the rootstock, the end products will be similar to those from conventional bred counterparts (as is the case with situation a. from the previous paragraph).

If RNAi-silencing rootstocks are used to assist breeding through gene-silencing in the scion (e.g. for reverse breeding or early flower induction; situation b. from the previous paragraph), the offspring can be regarded as non-genetically modified and is similar to offspring from its non-genetically modified counterpart. If however, the RNAi-silencing has led to methylation of the target DNA, the methylation-related phenotype may occasionally be stably inherited by the next sexual generation. Depending on the type of gene that is silenced the effect on the end products may be different. Therefore a case-by-case evaluation of the expected effects of a possible RNA-directed DNA methylation that is transmitted to the offspring is necessary.

In case grafting results in transmission of proteins and metabolites from the genetically modified rootstock to the scion (situation c. from the previous paragraph) this may result in a diversity of consequences, depending on the nature of the gene(s) used for the genetic modification. A case-by-case evaluation is necessary to evaluate these consequences and suitable baseline references should be used to compare the grafts with.

Because little is known about the transmission of metabolites and proteins (i.e. efficiency of transport, size and/or charge, distance, accumulation) of transported molecules from a rootstock to a scion more research is necessary on this subject before a general conclusion can be drawn.

Environmental consequences

One of the consequences of release of genetically modified crops into the environment is gene-flow from genetically modified crops to wild or cultivated cross-compatible plants through genetically modified pollen dispersal. Because in grafted plants the scion is usually the part that produces flowers, gene-flow of transgenes does not occur. In case RNAi-mediated silencing of the rootstock has led to RNA-directed DNA methylation of the target genes in the non-genetically modified scion, the methylation-related phenotype can occasionally be stably

inherited by the next sexual generation. If the methylation-related phenotype is expected to have an effect, the absence of silencing effects in the offspring should be determined, before introduction into the environment.

Another consequence is the result of interaction of the genetically modified rootstock with the soil environment. Depending on the nature of the genetic modification this may have an impact to soil organisms, leading to a change in soil biodiversity (see opinion paper by Lilley et al., 2006). As baseline grafted lines of the non-GM rootstock and scion can be used. As far as transmission of metabolites and proteins from rootstock to scion are concerned it very much depends on the nature and concentration of these compounds whether they pose a potential environmental problem. If the compounds are known and present in other plant species then one could use these as baseline to determine whether they are an environmental issue.

Consequences for food and feed safety

If in a graft of a genetically modified rootstock and a non-genetically modified scion the effects of the genetic modification are restricted to the rootstock, and products from the non-genetically modified scion are used for consumption, the consequences for food and feed safety will be comparable to the baseline.

If grafting leads to genetic modification-related alterations of the scion, as a result of transmission of RNAi-signals, proteins or metabolites, the consequences can be very diverse. The consequences are completely dependent on the nature of the genetic modification and can therefore not be considered in general terms. In such a situation a case-by-case consideration and comparison to a suitable baseline should be applied and different analytical methods, including proteomics and metabolomics should be used. As baseline grafted or non-grafted non-genetically modified plants can be used. Interspecific grafts may be an informative baseline reference. In a number of grafts combining different cucumber species, proteins that were specific to a rootstock species were also prominently present in the scion (Golecki et al., 1998). These proteins are not original to the plants of which the scions have been obtained. Zhang et al. (2008) show a widespread rootstock effect on gene expression of the scion in interspecific grafts of eggplant scions on tomato rootstock. Both references demonstrate the possible large-scale effects of grafting, even when combining non-genetically modified plants and emphasis the importance of selecting suitable baseline references.

Cisgenesis

Cisgenesis is the production of genetically modified plants using donor DNA from the species itself or from a cross compatible species. The newly introduced DNA is an unchanged natural genome fragment containing a gene of interest together with its own introns and regulatory sequences, like gene promoter and gene terminator DNA sequences. The introduced DNA is free of vector DNA, with the exception of T-DNA border sequences that are flanking the cisgenic DNA sequences.

Cisgenesis is a genetic modification method using donor gene sequences from the species itself or using donor genes from a natural crossable donor species (Schouten et al., 2006). These donor genes may for example code for disease resistance genes which are found in wild related species, but also beneficial alleles within the species may be transferred from one genotype to another. Because current developments in large-scale DNA sequencing techniques are leading to an exponentionally increase in the number of isolated and characterized genes, cisgenesis will have great potential as genetic modification method for future crop improvement.
In cisgenesis one or more native genes are used in the genetic modification, including their own introns and flanked by their 5'- and 3'- untranslated regions (UTR) and promoter and terminator sequence, all in their natural context (see Fig. 2).

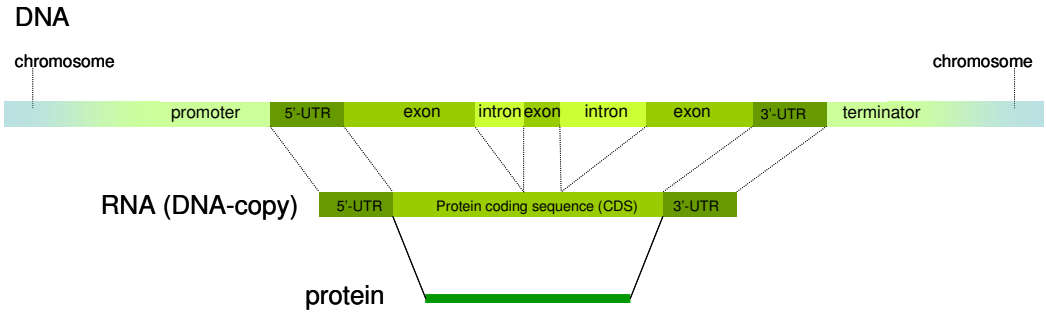

Figure 2. *Structure of a typical native gene. The gene is part of a chromosome and consists of a promoter and terminator, 5'- and 3'-untranslated regions (UTR) and exons and introns. The promoter and terminator are regulatory DNA sequences containing information for the expression of the gene in time, space and intensity. Before translation into protein, part of the gene is transcribed (copied) into an intermediate DNA-like molecule called RNA. During this process, all exons will be fused together and form the protein coding sequence.*

One important characteristic of cisgenesis is that the ultimate phenotype of the cisgenic plant also can be achieved by conventional breeding, but on a much longer term. In conventional breeding always a certain amount of linkage drag will occur (Jacobsen and Schouten, 2007).

Linkage drag is the simultaneous transfer of unwanted DNA sequences that are linked to the gene of interest, and the amount of linkage drag can be reduced by repeatedly back-crossing. This makes conventional breeding a difficult approach for improving crops with a long generation time, like fruit tree species, or crops with complex genetics (polyploidy, vegetatively propagated), like potato. The main difference between conventional breeding and cisgenesis is that in cisgenesis the newly introduced gene will be inserted as an extra gene copy at a random position in the target genome. The novel genomic context of such an insertion has often been claimed to be, at least partly, responsible for the observed variability in expression of the new gene, and is referred to as the 'position effect'. The random integration of the new gene can also have an effect on the expression of genes that are located on the recipient genome around the site of integration. The insertion itself can disrupt genes of the recipient, and enhancing or silencing elements that may be part of the promoter of the newly introduced gene may have an enhancing or silencing effect on the expression of genes of the recipient (Tani et al., 2004). Because the generation of genetically modified plants is a rather inefficient process, genetically modified plant production usually involves a selection step to separate genetically modified plants from plants that have not received donor-DNA. The most common way for selection is the use of antibiotic resistance genes or 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.

Potential application in current breeding programs

Cisgenesis is still in its research phase, but may find its first application in the coming 10 years. Current research in the Netherlands involves cisgenesis in apple and potato using disease resistance genes originating from wild crossable species.

Specific features of application of the technique

Cisgenic plants are enriched by the addition of one or more genes that originate from the species itself or from a cross-compatible species. In principle plants with a similar genotype and phenotype can be achieved by conventional breeding (Schouten et al., 2006), assuming that the gene introduced in the cisgenic plant has been transferred together with its own, full regulatory environment. Gene promoters are difficult to characterize and most often the length of the promoter is not well defined. They consist of an array of positive and negative regulatory elements which regulate gene expression in a concerted action. These elements are generally located within a DNA fragment of 500-1000 base pairs lying directly upstream of the gene (Barta et al., 2005), but promoters can have regulatory elements that are positioned several kilo bases away from the transcriptional start site (Goñi et al., 2007). Isolation of a cisgene with

insufficient promoter sequence length may result in deviation of gene expression when compared to the natural situation (Szankowski et al., 2009).

The insertion of donor DNA sequences in genetically modified plants brings about specific consequences that are related to the type and position of insertion of the donor sequence. It is frequently observed that multiple copies of the donor sequence are integrated into the recipient genome. These copies may be located at different loci, but also (direct or inverted) repeats of the donor sequence at a single locus are found frequently. The presence of multiple insertions can have a significant effect on the quality and level of expression of the introduced gene. However, many native genes are also present in duplicated forms in the plant genome. Because, the cisgene expression in plants with a single or low cisgene copy number usually (but not always) corresponds better to the baseline than the cisgene expression in plants with a high cisgene copy number, plants with a low copy number insertion will preferably be selected. Another integration-related consequence is the possible insertion of the cisgene into an existing gene. This creates the possibility that the inserted sequence becomes part of an existing open reading frame, resulting in the potential production of a new, chimeric protein. More likely the expression of the targeted gene will be disrupted due to insertional mutagenesis.

The random integration of the cisgene in the recipient genome can lead to the so-called position effect. This effect is used to describe the variation in expression of identical transgenes (or cisgenes or intragenes) that are inserted into different regions of a genome. The difference in expression is often due to enhancers that regulate the expression of neighboring genes in the recipient genome. These local enhancers can also influence the expression pattern of the newly introduced gene(s). The other way around, newly introduced gene may also influence the expression of genes of the recipient genome if they are located around the integration site. Originally, the position effect was described as a result of spontaneous structural chromosomal rearrangements which lead to a change in gene position and which in many cases can result in alteration of the gene expression (Papazova and Gecheff, 2003).

Another consequence is that, when making use of *Agrobacterium* mediated transformation, together with the cisgene(s) a minimal amount of foreign DNA will be transferred to the plant genome. This concerns the so-called right and left T-DNA border sequences (RB and LB resp.). These DNA sequences are only 24 bp long and are flanking the cisgene on the plant transformation vector. Usually, only part of the full length RB (namely 3 bp) and LB (21bp) are found back in genetically modified plants after *Agrobacterium*-mediated transformation. For the LB, however, frequently its complete sequence can be traced back, and if so, often together with some length of transformation vector DNA. In case the RB and LB sequences become part of an open reading frame (of a recipient gene), they can be translated into protein as part of a fusion protein. Such a situation is undesired and should be screened for by investigating the nature of the recipient genomic sequence that is flanking the T-DNA insert.

Methods for screening of presence/absence of gene constructs used

Standard PCR techniques are suitable to confirm the presence of genetic modification-related sequences into the cisgenic plant. PCR can also be used to check the presence of undesired DNA sequences originating from the plant transformation vector. Next to this, DNA-gel blot (also called Southern blot) analysis, using the complete T-DNA-vector backbone that was used for the genetic modification as probe, may give additional evidence for the cisgenic status of the selected lines. The number of cisgene copies inserted into the recipient genome can also be determined using DNA-gel blotting using the cisgene DNA sequence as probe, but one should be aware that also endogenous homologous gene copies will be detected using such a technique. In addition, quantitative PCR (qPCR) can be used to estimate the cisgene copy number. In order to investigate whether or not the cisgene has been inserted into a recipient gene, techniques like genome walking or inverse PCR (iPCR) can be used to amplify DNA sequences that are flanking the cisgene into the recipient genome. However the since the introduced T-DNA is identical to what might be present after introgression breeding this should not be a problem. It might however give an opportunity to get additional proof of the absence of T-DNA-vector backbone sequences and *Agrobacterium* chromosomal DNA sequences that may have been transferred together with the T-DNA.

Comparison of end products of new breeding techniques and conventional bred crops

The cisgenes already belong to the gene pool of the recipient plant and they can therefore also be transferred by conventional breeding. There are some differences however between end products obtained by cisgenesis and conventional breeding. In a cisgenic plant, the cisgene will be present as an extra gene copy. Next to this the cisgene will be inserted at a random position in the recipient genome, which might influence cisgene expression in quantity and quality when compared to the gene in its natural genomic context. This does normally not mean that there are inherent differences with regards to level and timing of expression. A baseline test to establish bandwidth of the expression of the cisgene should be able to tell whether this expression is significantly different from that in the original plant.

The insertion of a cisgene may result in a mutation in the recipient genome at the insert site (Forsbach et al., 2003). Such a mutation usually leads to a disruption of gene function in the recipient genome and can thereby induce phenotypic effects. Next to this, the newly introduced gene may also influence the expression of genes of the recipient genome if they are located around the integration site. However, both effects of cisgene integration are natural phenomena occurring during transposon transition (Greco et al., 2001) and translocation breeding, respectively (Papazova and Gecheff, 2003).

Finally, together with the cisgene, some small non-coding sequences originating from the transformation vector, like T-DNA border sequences, will be transferred to the recipient genome. These sequences may become part of a open reading frame in case the T-DNA has been inserted into a recipient gene, and lead to new chimeric proteins.

Environmental consequences

The cisgenes already belong to the gene pool of the recipient plant and they can therefore also be transferred by conventional breeding. There is however a chance that the expression of the cisgene differs from the expression of the endogenous gene when it is in its natural genomic position, e.g. through the described position effect, and this may lead to phenotype differences. Because the position effect is a naturally occurring phenomenon, in cisgenic plants this is not expected to have consequences for the environment. Of course the cisgene expression should be checked and compared with the baseline. Conventionally bred plants and related species from which cisgenes have been isolated can be used as baseline.

The random integration process may cause cisgenes to integrate into genes of the recipient genome. This would most likely lead to insertional mutagenesis of the gene in which the cisgene has integrated. Part of the recipient genome that is flanking the inserted T-DNA should be sequenced to show that the cisgene has integrated outside genes of the recipient genome.

As a consequence of the transformation method used, fragments of the RB and LB will be integrated into the plant genome together with the cisgene. These short sequences are by nature non-coding and are unlikely to have a phenotypic effect (Schouten et al., 2006).

In case the variation of cisgene expression is within the range of expression variation of the corresponding gene in its natural genomic context, and when no genes of the recipient have been mutated as a result of cisgene integration, and when the RB and LB have not become part of an open reading frame, the cisgenic plants are similar to conventionally bred plants. Such conventionally bred plants can serve as baseline.

Consequences for food and feed safety

The aim of cisgenesis is to improve existing elite crop varieties with new valuable characteristics. For this, a single or a few genes from crossable species are introduced into an elite variety with a safe history of food. There is however a chance that the expression of the cisgene differs from the expression of the endogenous gene when it is in its natural genomic position, e.g. through the described position effect, and this may lead to phenotype differences. Because the position effect is a naturally occurring phenomenon, in cisgenic plants this is not expected to have consequences for the environment. Of course the cisgene expression should be checked and compared with the baseline. Conventionally bred plants and related species from which cisgenes have been isolated can be used as baseline.

The random integration process may cause cisgenes to integrate into genes of the recipient genome. This would most likely lead to insertional mutagenesis of the gene in which the cisgene has integrated. Part of the recipient genome that is flanking the inserted T-DNA should be sequenced to show that the cisgene has integrated outside genes of the recipient genome.
As a consequence of the transformation method used, fragments of the RB and LB will be integrated into the plant genome together with the cisgene. These short sequences are by nature non-coding and are unlikely to have a phenotypic effect (Schouten et al., 2006).
In case the variation of cisgene expression is within the range of expression variation of the corresponding gene in its natural genomic context, and when no genes of the recipient have been mutated as a result of cisgene integration, and when the RB and LB have not become part of an open reading frame, the cisgenic plants are similar to conventionally bred plants. Such conventionally bred plants can serve as baseline.

Intragenesis

Intragenesis is the production of genetically modified plants using donor DNA from the species itself or from a cross compatible species. The difference with cisgenesis is that intragenesis allows the creation of new combinations of DNA fragments which are all originating from the species itself or from a cross compatible species. In intragenesis also the transformation vector itself can be composed of functional DNA fragments from the genome of the target crop species.

Technical description of the technique

Like cisgenesis, intragenesis is a genetic modification method using donor gene sequences from the species itself or using donor genes from a natural crossable donor species. The difference with cisgenesis is that in intragenesis new genes can be created 'in vitro' by combining functional genetic elements such as promoters, coding parts (with or without introns) and terminators of natural genes, and insert this new chimeric gene into existing varieties (Rommens et al., 2004, 2006, 2007; Conner et al., 2007; Schouten and Jacobsen 2008). Intragenesis also allows the use of inverted DNA repeats for RNA interference (RNAi) with the aim of gene silencing.

Rommens et al. (2004) showed that functional T-DNA-like sequences are present in plant genomes (called P-DNA). These P-DNAs can replace the *Agrobacterium*-derived T-DNA sequences which are an essential part of the plant transformation vectors. Rommens et al. (2006) introduced the term all-native DNA transformation for transformation using intragenes combined with species-specific P-DNA so that exclusively native DNA was inserted into the plant genome. For all-native DNA transformation, Rommens et al. (2006, 2007) combined the P-DNA with an *Agrobacterium* binary vector. Conner et al. (2007) constructed species-specific transformation vectors that are completely composed of functional plant DNA fragments from the genome of the recipient crop species. They argued that using vectors derived entirely from plant sequences would give transformed plants that never do contain foreign DNA, regardless of whether transformation events extend beyond the T (or P)-DNA region.

Differently than in cisgenesis, the ultimate phenotype of the intragenic plant cannot always be achieved by conventional breeding. If for example new combinations of regulatory elements and protein coding sequences have been created, the expression level and pattern of the novel gene combination may differ from the natural situation. In case intragenesis aims at silencing of a single gene through RNAi, similar phenotypes can be obtained by mutation breeding.

Potential application in current breeding programs

In the USA the production of intragenic potato plants, in which three different genes were silenced through RNAi, was published (Rommens et al., 2006). This was achieved by combining functional DNA fragments of at least 7 different potato genes in a transformation construct leading to the production of a quality-enhanced potato. In Canada intragenesis is currently being used to develop non-browning apples by transforming them with RNAi silencing constructs against the apple polyphenol oxidase gene (www.okanaganbiotechnology.com). Another ongoing intragenesis project in New Zealand develops drought-tolerant ryegrass (*Lolium perenne*) that over-expresses a native salt-tolerance gene (www.isb.vt.edu/articles/aug0601.htm;). These examples show that intragenesis is developing towards a method that may be applied in the near future, at least outside Europe.

Specific features of application of the technique

The possible novel combinations of functional genetic elements that are present in intragenic plants do not exist in nature and it is unlikely that they would arise spontaneously or as a result of conventional breeding. Depending on the regulatory and coding sequences used for the genetic modification, gene expression may deviate considerably in level and pattern from the natural situation as found in conventionally bred crops. Therefore, a case by case evaluation is necessary to discuss the consequences of the technique applied, especially when the intention of intragenesis is to change the expression level and pattern of native genes.

In case intragenesis is aimed at silencing of native genes, a similar phenotype may be obtained through mutation breeding. In general, consequences of silencing as a result of intragenesis are not different from those of mutation breeding leading to knock-out mutations.

The insertion of donor DNA sequences in genetically modified plants brings about specific consequences that are related to the type and position of insertion of the donor sequence. It is frequently observed that multiple copies of the donor sequence are integrated into the recipient genome. These copies may be located at different loci, but also (direct or inverted) repeats of the donor sequence at a single locus are found frequently. The presence of multiple insertions can have a significant effect on the quality and level of expression of the introduced gene. However, many native genes are also present in duplicated forms in the plant genome. Because, the intragene expression in plants with a single or low cisgene copy number usually (but not always) corresponds better to the baseline than the intragene expression in plants with a high intragene copy number, plants with a low copy number insertion will preferably be selected. Another integration-related consequence is the possible insertion of the intragene into an existing gene. This creates the possibility that the inserted sequence becomes part of an existing open reading frame, resulting in the potential production of a new, chimeric protein. More likely the targeted gene will be disrupted due to insertional mutagenesis.

The random integration of the cisgene in the recipient genome can lead to the so-called position effect. This effect is used to describe the variation in expression of identical transgenes (or cisgenes or intragenes) that are inserted into different regions of a genome. The difference in expression is often due to enhancers that regulate the expression of neighboring genes in the recipient genome. These local enhancers can also the influence expression pattern of the newly introduced gene(s). The other way around, newly introduced gene may also influence the expression of genes of the recipient genome if they are located around the integration site. Originally, the position effect was described as a result of spontaneous structural chromosomal rearrangements which lead to a change in gene position and which in many cases can result in alteration of the gene expression (Papazova and Gecheff, 2003).
Like in *Agrobacterium*-mediated transformation using T-DNA vectors, by the use of P-DNA vectors, DNA sequence that flank the intragene will also integrate into the plant genome. Because the P-DNA is originating from the species itself, no consequences are expected from integration of such sequences. The resulting recombinations are similar to those that arise by genome rearrangements caused by mutagenesis.

Methods for screening of presence/absence of gene constructs used
Standard PCR techniques are suitable to confirm the presence of genetic modification-related sequences into the intragenic plant. The number of intragene copies inserted into the recipient genome can be determined using DNA-gel blot (also called Southern blot) analysis using the intragenic DNA sequence as probe, but one should be aware that also endogenous homologous gene copies may be detected using such a technique. In addition, quantitative PCR (qPCR) can be used to estimate the intragene copy number. In order to investigate whether or not the intragene has been inserted into a recipient gene, techniques like genome walking or inverse PCR (iPCR) can be used to amplify DNA sequences that are flanking the P-DNA into the recipient genome. This will show if the introduced P-DNA has been inserted into a recipient gene or not. Furthermore, this gives an additional proof of the absence of vector backbone sequences (in case the vector has not been derived entirely from DNA of the genome of the recipient species) and *Agrobacterium* chromosomal DNA sequences that may have been transferred together with the P-DNA.

Comparison of end products of new breeding techniques and conventional bred crops
The genes that are inserted by intragenesis are novel combinations of functional genetic elements, but all from native origin. Because of the new combinations, the expression of the intragene may deviate from the natural situation as found in baseline references. Because this new combinations usually do not exist in the natural situation, no general comparison with conventionally bred crops can be performed, and a case-by-case study is required. Next to this

aspects like copy number of the inserted intragene and the position of integration in the recipient genome may have an additional effect on variability of intragene expression.
In case intragenesis is aimed at silencing of single endogenous genes, the end products may be compared to knock-out mutants obtained by mutation breeding.
The insertion of an intragene may result in a mutation in the recipient genome at the site of insertion (Forsbach et al., 2003). Such a mutation usually leads to a disruption of gene function in the recipient genome and can thereby induce phenotypic effects. Next to this, the newly introduced gene may also influence the expression of genes of the recipient genome if they are located around the integration site. However, both effects of intragene integration are natural phenomena occurring during transposon transition (Greco et al., 2001) and translocation breeding, respectively (Papazova and Gecheff, 2003).

Environmental consequences

The random integration process may cause intragenes to integrate into genes of the recipient genome. This would most likely lead to insertional mutagenesis of the gene in which the intragene has integrated. Part of the recipient genome that is flanking the inserted T-DNA should be sequenced to show that the intragene has integrated outside genes of the recipient genome.
Because of their recombinant nature, the expression of the intragenes is expected not always to correspond to the expression of the native corresponding genes in their natural genomic position. Next to this there may be variability in expression of the intragene caused by the position effect. Depending on the nature of the intragenes and the impact of the position effect, this may have different consequences for the environment when compared to the baseline. This means that for intragenesis no general statement about consequences for the environment can be made and a case-by-case evaluation is required. As baseline conventionally bred plants belonging to the same, or sexual compatible species can be used.
If intragenesis is specifically aimed at silencing of single endogenous genes, the intragenic plants may be comparable to plants with knock-out mutations obtained by mutation breeding. Such plants can be used as baseline. In general, the consequences of intragenesis aimed at gene-silencing of a single gene will be similar to consequences of mutation breeding, assuming that insertion of the intragene has not led to insertional mutagenesis.

Consequences for food and feed safety

For intragenesis new combinations of native functional genetic elements are made which lead to chimeric genes that do not exist in nature. These new genes may have expression levels and patterns that do not correspond to that of the native gene. Next to this the position effect may have an additional effect on the intragene expression. The consequences for food and feed

safety of this deviation in gene expression should be evaluated in a case-by-case study and compared to the baseline. As baseline products from conventionally bred plants can be used. If intragenesis was used for the silencing of a single native gene, the consequences for food and feed safety are in general similar to that of crops obtained by mutation breeding in which the same gene is knocked-out. Such plants from mutation breeding programs can serve as baseline. The random integration of intragenes may have an effect on the expression of genes of the recipient genome. This phenomenon is however also expected when translocation breeding, a traditional breeding method, is applied. To investigate whether or not the intragene has been inserted into a recipient gene, and has become part of an open reading frame,, the part of the recipient genome that is flanking the inserted P-DNA site can be sequenced..

Oligonucleotide-mediated mutation induction

Oligonucleotide-mediated mutation induction is a site-specific gene modification system that makes precise changes in a gene sequence without the incorporation of genes that are foreign to the species.

Technical description of the technique

Oligonucleotide-mediated mutation induction is a method to generate precise gene mutations on a targeted plant genome locus. It makes use of chemically synthesized chimeric oligonucleotides, which are small (approximately 70 nucleotides) nucleic acids composed of both DNA and modified RNA nucleotides, to induce nucleotide substitutions, insertions or deletions in genomic sequences.

The oligonucleotides are introduced using particle bombardment of plant tissue or electroporation of protoplasts. In the plant cell the oligonucleotides are believed to hybridize at the targeted gene location to create a mismatched base-pair. This mismatched base-pair induces precise correction (replace, insert or delete) of the designated nucleotide by the cell's own natural gene repair system. Once the correction process is complete the oligonucleotides are degraded, so there will be no integration of foreign DNA into the plant genome. Mutation of the gene coding sequence can be aimed at knocking out gene function by changing an amino acid codon into an early stop codon, or by introducing a reading-frame shift mutation. Alternatively, the functionality of a gene can be altered by oligonucleotide-mediated mutation that lead to amino acid substitutions. Finally, gene promoter sequences can be the target of oligonucleotide-mediated mutation induction with the aim to alter the gene expression properties of the gene.

Potential application in current breeding programs

So far, oligonucleotide-mediated mutation induction has only been described for mutations that lead to amino acid substitutions into the acetolactate synthase (*als*) gene resulting in an herbicide-resistant phenotype (Beetham et al., 1999; Kochevenko and Willmitzer, 2003; Okuzaki et al., 2004) or described for the repair of a mutated reporter gene that was introduced into a transgenic plant (Dong et al., 2006). Recently, in January 2009, BASF announced the development of herbicide tolerant *Brassica* lines using oligo-mediated mutation induction of the *als* gene. At the moment, the use of oligonucleotide-mediated mutation without the possibility of selection is limited by its low efficiency. This makes it unsuitable for practical applications other than introduction of herbicide resistance.

Specific features of application of the technique

Oligonucleotide-mediated mutation induction is a promising approach for knocking out or adapting gene function in crops. The method aims at precise and specific mutations of an endogenous gene sequence without the integration of foreign DNA into the plant genome. Kochevenko and Willmitzer (2003) show however that in addition to the intended mutations, also semi-targeted mutations were observed. These mutations were observed in nucleotides direct adjacent to the intended one.
Mutants obtained by the described techniques can in principle also be obtained through genome-wide mutation induction methods (using ionizing radiation or chemical mutagens), which have a long history of application in plant breeding. For this reason no new consequences are expected when compared to accepted methods for mutation induction.
The methods described for delivery of the chimeric oligonucleotides into the plants cells are also common methods used for stable transformation (i.e. stable genomic integration of introduced DNA) of plants (Newell, 2000). Therefore it is likely that the chimeric oligonucleotides may also integrate in the plant genome at a certain frequency. Although it is noted in the publications that the chimeric oligonucleotides are expected to be degraded after mutation induction, this was not demonstrated in any of the publications describing oligonucleotide-mediated mutation induction. Plants obtained by oligonucleotide-mediated mutation induction should therefore be screened for absence of integrated chimeric oligonucleotides.

Methods for screening of presence/absence of gene constructs used
The presence of the intended mutations can be checked by DNA-sequencing of the known mutated gene fragment. DNA-gel (Southern) blot analysis can be used to screen for possible incorporation of oligonucleotides into the plant genome.

Comparison of end products of new breeding techniques and conventional bred crops
Mutants obtained by the described techniques can in principle also be obtained through mutation breeding (using ionizing radiation or chemical mutagens), which has a long history of application in plant breeding (Ahloowalia et al., 2004). End-products from plants produced by oligonucleotide-mediated mutation induction are therefore similar to plants obtained through mutation breeding. Like in mutation breeding, in oligonucleotide-mediated mutation induction the occurrence of semi-targeted mutations has been reported (Kochevenko and Willmitzer, 2003). Plants selected in mutation breeding are always tested for undesired traits (caused by non-targeted mutations) before varieties are market released, and the same should be applicable to plants obtained through oligo-mediated mutation induction.

Environmental consequences

Introduction of plants that are obtained through oligonucleotide-mediated mutation induction in the environment has no additional consequences to introduction of plants generated by mutation breeding. Of course such plants should be free of incorporated oligonucleotides that are used for the mutation induction. Such mutants are a suitable base line to compare oligonucleotide-mediated mutation-derived plants with. Plants that are selected in mutation breeding are always tested for undesired traits that may have resulted from the mutation treatment, before they are released. Plants obtained through oligonucleotide-mediated mutation induction should therefore be tested in the same way.

Consequences for food and feed safety

Plants that are obtained through oligonucleotide-mediated mutation induction have a single change in a target gene that could also be obtained through mutation breeding. Plants obtained by mutation breeding are therefore a suitable base line. Supposing that the mutated plants are free of incorporated oligonucleotides, the consequences for food and feed safety are similar to those of the baseline. Plants that are selected in mutation breeding are always tested for undesired traits that may have resulted from the mutation treatment, before they are released. Plants obtained through oligonucleotide-mediated mutation induction should therefore be tested in the same way.

Conclusions

The new plant breeding techniques described in this report share as common feature that they all involve a genetic modification step, but in the end all lead to end products (plants or plant parts) that are free of genes that are foreign to the species. Because of the application of the genetic modification step, plants produced by the new plant breeding techniques all fall under the current European Directive 2001/18/EC, even if the plants are completely free of any DNA sequence that was used for the genetic modification. This fact leads to a request for modernization of the current EU regulations for the release of genetically modified plants. The safety assessment that is required for the admission procedure of any genetically modified organism is costly and time-consuming. Moreover the absence of foreign DNA sequences brings about the extreme difficulty and in some cases the inability to indicate the end products as being genetically modified. This may complicate the enforcement of the regulations.

To support the modernization of the current EU regulations, this report describes a technical-scientific approach to assess possible consequences of new breeding techniques for the environment and human and animal health. For the discussion of the consequences, the new plant breeding techniques are compared to a baseline, which is defined by references, for example similar plants, but bred according to conventional breeding techniques. The baseline covers the 'natural' situation in its full bandwidth. Possible baselines for each technique are summarized in Table 2.

For all plants or plant products which are a result of the new plant breeding techniques it is clear that general precautions or actions have to be undertaken to prove that the final product is free of agrobacteria and sequences that are foreign to the species. As represented in see Table 2, this can entail to performing PCR tests or DNA gel blot (Southern blot) hybridization analysis.

Some methods aim at transient gene-silencing by RNAi. One of the lesser understood aspects of RNAi is that it occasionally can induce RNA-directed DNA methylation.
Methylation may result in both silencing and up regulation of the target gene expression.
In some cases a methylation-related changed phenotype was even found to be stably inherited by the next sexual generation. Therefore, the expression of the genes that were intended to be silenced transiently should be carefully evaluated and compared to the baseline when RNAi is applied.
Four different classes of techniques are described in detail in this report. For each new plant breeding technique the consequences for the environment and for food and feed safety are discussed. The consequences of each technique are summarized in Table 2.

The first class of new plant breeding techniques entails different techniques (agroinfiltration, virus induced gene silencing (VIGS), reverse breeding and accelerated breeding following induction of early flowering) in which genetic modification is used as a tool to facilitate breeding. Plants and products from this first class do not contain any genetic material that was used for the initial genetic modification. Therefore, plants and products obtained through these new plant breeding techniques are similar to the baseline, which are traditionally bred plants, and it follows that the consequences for the environment and food and feed safety do not differ from that of traditionally bred plants. This fact justifies the exemption of these plants from the European regulations for genetically modified organisms.

The second class of new plant breeding techniques, describes the production of chimeric, partially genetically modified plants. These plants are obtained by combining genetically modified and non-genetically modified plants by grafting. At such a graft, products (like fruits or flowers) can be produced on the non-genetically modified plant that is grown on a genetically modified root stock. Although the non-genetically modified part of the grafted plants do not contain any new genetic material, RNA molecules, proteins and metabolites that are transported from the genetically modified rootstock may be present. Therefore, plants and products obtained in this class may differ from the baseline, and no general conclusion with respect to consequences for the environment and food and feed safety can be made. Here a case-by case evaluation is required.

The third class of new plant breeding techniques uses genetic modification as a direct tool to introduce new, but native characteristics to a plant (cisgenesis, intragenesis). The introduced DNA is originating from the species itself or from a cross-compatible species.
When making use of Agrobacterium mediated transformation, together with the native gene sequences, a minimal amount of foreign DNA will be transferred to the plant genome. This concerns the so-called right and left T-DNA border sequences. In some cases these sequences may become part of an open reading frame if the T-DNA has been inserted into a recipient gene, and this may lead to new chimeric proteins. Such a situation is undesired and should be screened for by investigating the nature of the flanking recipient genomic sequence. Using Agrobacterium-mediated genetic modification, the cis- or intragenes sequences will integrate at a random position in the recipient genome. This random position may influence in expression of the cis- or intragene through a phenomenon called position effect, and this may lead to phenotype differences. Because the position effect is a naturally occurring phenomenon which is also found as a result of introgression breeding, the position effect is not expecting to have consequences for the environment of food and feed safety. Of course the expression of the

newly introduced genes should be checked and compared with the baseline. Next to this, the newly introduced gene may also influence the expression of genes of the recipient genome if they are located around the integration site. However, also this is a natural phenomenon that may occur during translocation breeding and is therefore also not expected to have consequences for the environment of food and feed safety.
In case the integration of the new genes was proven to be outside genes of the recipient genome and the introduced genes show an expression that corresponds to the baseline, such cisgenic and intragenic plants are regarded as similar to the baseline, also in terms of environmental safety and food and feed safety. If it is also proven that the final product is free of agrobacteria, virus (when used during the process) and sequences that are foreign to the species, this justifies the exemption of such cisgenic and intragenic plants from the European regulations for genetically modified organisms. In general however, intragenesis is often aimed at changing the expression of native genes. In case the intragene expression deviates from that of the baseline, additional studies are required to assess the environmental and food and feed safety.

The last class of new breeding techniques considered in this report concerns techniques where genetic modification is used as a tool to make specific mutations to native genes, for example using oligo-mediated mutation induction. Plants and products from this last class of new plant breeding techniques do not contain any genetic material that was used for the initial genetic modification. Plants and products obtained through this class of new plant breeding techniques are therefore similar to the plants obtained by traditional mutation breeding, which are used as baseline references, and it follows that the consequences for the environment and food and feed safety do not differ from that of traditionally mutated plants. The fact that plants from this class are as safe as traditionally bred plants, justifies the exemption of these plants from the European regulations for genetically modified organisms.

	Is end product completely free of DNA-sequences related to the genetic modification (GM) used?			Environmental consequences		Consequences for food and feed safety	
New plant breeding method	**GM-free end product?**	**Qualification for GM-free status of end-product**	**Screening method**	**Baseline**	**Are consequences similar to baseline**	**Baseline**	**Are consequences similar to baseline**
Agroinfiltration	Yes	*A. tum*-free	Enrichment of *A. tum* + PCR	Original plants before agroinfiltration	Yes	Original plants before agroinfiltration	Yes
		T-DNA-free[1]	PCR; Southern blot				
VIGS	Yes	*A. tum*-free	Enrichment of *A. tum* + PCR	Original plants before application of VIGS	Yes	Original plants before application of VIGS	Yes
		T-DNA-free[1]	PCR; Southern blot				
		Virus-free	PCR; Southern blot				
Reverse breeding	Yes	T-DNA-free[1]	PCR; Southern blot	Parental lines obtained by conventional breeding	Yes	Original heterozygous line used for reverse breeding; Conventional bred F1 hybrids	Yes
Accelerated flowering	Yes	T-DNA-free[1]	PCR; Southern blot	Plants obtained by conventional breeding	Yes	Plants obtained by conventional breeding	Yes
Grafting non-GM on GM	Yes[2]	-	-	Non-GM grafts;	Case-specific[3]	Non-GM grafts; Interspecific grafts	Case-specific
Cisgenesis	No	GM sequence is of native origin		Conventionally bred lines; Plant from which (cis)genes have been isolated	Yes	Conventionally bred lines; Plant from which (cis)genes have been isolated	Yes
		A. tum-free	Enrichment of *A. tum* + PCR				
		Vector DNA-free[1]	PCR; Southern blot				
Intragenesis	No	GM sequence is of native origin		Conventionally bred lines; plants from which native DNA sequences have been isolated; mutants from mutation breeding	Case-specific	Conventionally bred lines; plants from which native DNA sequences have been isolated; mutants from mutation breeding	Case-specific
		A. tum-free	Enrichment of *A. tum* + PCR				
		Vector DNA-free[1]	PCR; Southern blot				
Intragenesis for gene-silencing (RNAi)	No	GM sequence is of native origin		Knock-out mutants from mutation breeding (in case of silencing of a single gene)	Yes	Knock-out mutants from mutation breeding (in case of silencing of a single gene)	Yes
		A. tum-free	Enrichment of *A. tum* + PCR				
		Vector DNA-free[1]	PCR; Southern blot				
Oligo-induced mutation induction	Yes	Oligo-free	Southern blot	(Knock-out) mutants from mutation breeding	Yes	(Knock-out) mutants from mutation breeding	Yes

Table 2. *Global overview of consequences of different new plant breeding techniques for the environment and for food and feed safety.*
It is assumed that for the plants still containing GM-related sequences, molecular characterization, like a check for absence of T-DNA vector backbone sequences, characterization of the copy number of the insertion, characterization of the position of the insertion in the recipient genome and analysis of possible formation of new open reading frames has been performed. GM, Genetic Modification. [1] *Including Agrobacterium chromosomal DNA that may be associated with the T-DNA or vector DNA.* [2] *The end products are harvested from the non-GM part.* [3] *E.g. interaction of GM rootstock with soil organisms*

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