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Cisgenesis

A report on the practical consequences of the application of novel techniques in plant breeding

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Summary

The technical and scientific progress in plant breeding, plant transformation technologies and rapidly evolving new concepts beyond the usual approaches led to discussions whether the application of new plant breeding techniques results in plants defined as “genetically modified organism” (GMO) as laid down in the relevant EU legislation. This uncertainty raises a number of questions of fundamental and practical relevance to regulators, stakeholders, and consumers. The European Commission has launched a number of activities to clarify questions arising from breaking innovations in plant breeding. At request of the Competent Authorities under Directive 2001/18/EC a working group analysed selected new breeding techniques taking into consideration relevant terms and issues. Thus, the experts evaluated whether the techniques result in genetic modification and whether resulting organisms fall within the scope of the EU GMO legislation. The Joint Research Centre (JRC), the Institute for Prospective Technological Studies (IPTS) and the Institute for Health and Consumer Protection (IHCP) published a joint report on the state-of-the art and prospects for commercial development concerning new plant breeding techniques. The European Food Safety Authority (EFSA) published a scientific opinion related to the safety assessment of plants developed through cisgenesis and intragenesis.

The overall objective of the present report is to provide an overview on the application of new techniques in plant breeding and the evaluation of potential consequences in different legal scenarios concerning detection, traceability, labelling, and risk assessment. This report focuses on the following techniques: cisgenesis, oligonucleotide-directed mutagenesis (ODM), zinc-finger nucleases (ZFN) and agroinfiltration. In addition, potential combinations of the listed techniques are identified. The report aims to analyse the state-of-the-art concerning the application of the selected new techniques in plant breeding, in particular regarding plant species and transformation methods, to assess breeding goals of the modifications, targeted traits, and anticipated developments, and how the alterations can be detected. Furthermore, current risk assessment practices are discussed, with respect to the steps of the current procedures as set out by EFSA. Practical consequences (case studies/model procedures) regarding the plants under investigation are highlighted. Finally, the report provides recommendations taking into consideration different scenarios related to the GM/non GM status of plants developed through new techniques. This assignment does not include a classification of the new techniques concerning their GMO status.

Some new techniques have already been adopted by the breeding sector in their research and development. However, to date no products developed through the application of new techniques have been placed on the market. How soon such products will be commercialised depends on many factors, including legislative decisions on the European level. The present report may thus only be indicative of the anticipated developments and their practical consequences.

Within the risk assessment of the plants under investigation, a thorough molecular characterisation is necessary proving that only the intended modification has taken place, and that neither unintended effects nor unwished insertions have occurred. The minimum requirement is thus a molecular analysis and documentation which in detail characterises the sequence of the target site and the flanking sequences. This, together with a phenotypic analysis provides the basis for further (case-by-case) decisions concerning the necessary elements in the risk assessment procedure.

In most cases, the methods of choice used to deliver DNA into plant cells are the same as in transgenesis. Cisgenic plants are usually produced through *Agrobacterium*-mediated transformation. This method is prone to cause mutations like deletions and rearrangements within the plant genomic DNA. It has to be taken into account that recombinant DNA technology is different from meiotic recombination, and unintended effects resulting from the transformation process have to be minimised to the extent possible.

With regard to the available gene pool, only genes from sexually compatible species are transferred into cisgenic plants, whereas in site-directed mutagenesis endogenous genes are specifically targeted. Hence, elements of the risk assessment procedure, e.g. environmental risks caused by gene transfer, may be reconsidered. The fact that a modified gene and, if applicable, a newly expressed protein stem from the same or cross-compatible species or have been directly modified in the genome lowers the chances that potential adverse effects to the environment occur. If a gene has already been present in the (cross-compatible) population, a number of risks associated with transgenic plants are presumably not relevant. Furthermore, it is possible that the application of new techniques results in plants not substantially different in their characteristics from those bred traditionally. It is reasonable then to anticipate that the risks they pose for human and animal health are similar, and the food and feed safety assessment may be conducted accordingly.

In conclusion, the basis to assess potential risks arising from plants developed through new techniques and derived food and feed is similar to that applying to GM plants. Thus, the respective EFSA Guidance Documents are applicable for the evaluation of food and feed products and for performing the environmental

risk assessment. Depending on the characteristics of the plant under investigation the data requirements may be reconsidered case-by-case.

Plants developed through new techniques frequently display characteristics that affect laboratory testing and detection in the supply chains. Regardless of the applied technique, it is necessary to know the site of the genetic modification in the genome. Given this information, detection methods may generally be developed for all plants derived from the use of novel techniques. Cisgenic plants harbour a unique combination of the inserted and the flanking sequences in the plant genome, which allows for event-specific, unequivocal detection and quantification. Some genomic modifications are expected to be indistinguishable from those occurring during traditional plant breeding. For instance point mutations induced by ODM or ZFN-1 techniques may be detected but it is not possible to identify the origin of the mutation. If ZFN type-3 is combined with cisgenesis to replace an endogenous sequence with a highly similar one from a cross-compatible species it is likely difficult to develop suitable detection methods.

One key aspect for labelling of a GMO commodity or product is the availability of reliable protocols for unequivocal detection and quantification. Thresholds for the adventitious or technically unavoidable presence of authorised GMOs have been established, and the labelling of products (material) consisting, containing or produced from GMOs is currently based on the unequivocal quantification of the presence of an authorised GMO. It can be difficult to quantify the presence of plant material developed through new techniques, and thus to follow the labelling provisions according to the current GMO legislation. This is likely the case for ODM and ZFN-1 leading to point mutations, for which it is not easy to develop an unambiguous quantification method.

In case of authorised GMOs, commonly their presence is traced by suitable detection methods, which are also necessary for surveillance purposes. Traceability is largely based on documentation and general traceability systems are foreseen in the major supply chains, food, feed and seed. Independent of the GMO status, the application of a new technique can easily be traced back by appropriate documentation if the applicant provides the adequate information during the variety registration process. Specific legislative measures have been set into force concerning the traceability of GMOs; their applicability will depend on the classification (GM/non-GM status) of plant varieties developed through new breeding techniques.

Before a plant harbouring a GMO event may be used to develop a new variety, the event has to be approved by the European Commission. In addition, GM varieties are clearly identified in the Common Catalogues of varieties by means of footnotes indicating the authorisation of an event with the relevant

Commission Decision. At the beginning of the variety registration process, the applicant has to provide a written confirmation whether the variety is GM or not. In seed production, general traceability due to the requirements of the European seed certification system is given. Seed lots are requested to be certified, unique lot identity numbers and defined duties to keep records throughout the seed production, processing and distribution processes are foreseen. If seed is produced from a GM variety, the clear labelling of the seed as GM throughout the seed production process is mandatory. Food and feed products produced from GM seed are subject to the national and European regulatory frameworks for GMOs.

The classification of the resulting plants (GM/non-GM) is identified as crucial in relation to the practical consequences of the application of new techniques in plant breeding. The status of the plants determines the legislative measures applying and consequently risk assessment, detection and traceability.

Zusammenfassung

Die technischen und wissenschaftlichen Fortschritte in der Pflanzenzüchtung, Pflanzentransformationstechnologien und sich schnell entwickelnde neue Konzepte außerhalb der üblichen Ansätze führten zu Diskussionen, ob die aus der Anwendung neuer Züchtungsverfahren hervorgehenden Pflanzen als "genetisch veränderter Organismus" (GVO) gemäß den einschlägigen EU-Rechtsvorschriften zu definieren sind. Diese Unsicherheit wirft eine Reihe von Fragen von grundsätzlicher und praktischer Relevanz für Regulatoren, Interessensgruppen und Verbraucher auf. Die Europäische Kommission hat eine Reihe von Aktivitäten gestartet, um Fragen aus zukunftsweisenden Innovationen in der Pflanzenzüchtung zu klären. Auf Ersuchen der zuständigen Behörden gemäß der Richtlinie 2001/18/EG analysierte eine Arbeitsgruppe ausgewählte neue Züchtungsmethoden unter Berücksichtigung der einschlägigen Begriffe und Themen. Die Experten bewerteten, ob die Techniken als das Ergebnis einer genetischen Veränderung anzusehen sind und ob die entstehenden Organismen unter die EU-GVO-Gesetzgebung fallen. Das Joint Research Centre (JRC), das Institute for Prospective Technological Studies (IPTS) sowie das Institute for Health and Consumer Protection (IHCP) veröffentlichten einen gemeinsamen Bericht über den Stand der Technik und Perspektiven für die wirtschaftliche Entwicklung in Bezug auf neue Züchtungsverfahren. Die Europäische Behörde für Lebensmittelsicherheit (EFSA) veröffentlichte ein wissenschaftliches Gutachten in Bezug auf die Bewertung der Sicherheit von Pflanzen, die durch Cisgenetik und Intragenetik entwickelt wurden.

Das Ziel des vorliegenden Berichts ist es, einen Überblick über die Anwendung der neuen Techniken in der Pflanzenzüchtung und der Evaluierung der potenziellen Folgen in unterschiedlichen rechtlichen Szenarien bezüglich Nachweisbarkeit, Rückverfolgbarkeit, Kennzeichnung und Risikobewertung zu geben. Dieser Bericht konzentriert sich auf die folgenden Techniken: Cisgenetik, Oligonukleotid-gerichtete Mutagenese (ODM), Zink-Finger-Nukleasen (ZFN) und Agroinfiltration. Darüber hinaus wurden mögliche Kombinationen der angeführten Techniken analysiert. Der Bericht zielt darauf ab, den Stand der Technik der Anwendung ausgewählter neuer Techniken in der Pflanzenzüchtung, insbesondere im Hinblick auf Pflanzenarten und Methoden der Transformation, die Zuchtziele der Modifikationen, Zielmerkmale und erwartete Entwicklungen, sowie die Nachweisbarkeit der Änderungen zu untersuchen. Darüber hinaus werden aktuelle Methoden der Risikobewertung diskutiert, mit Bezug auf die von der EFSA festgelegten Schritte der aktuellen Verfahren. Praktische Konsequenzen (Fallstudien bzw. Modellverfahren) in Bezug auf die untersuchten Pflanzen werden hervorgehoben. Schließlich bietet der Bericht Empfehlungen unter Berücksichtigung unterschiedlicher Szenarien

im Zusammenhang mit dem GV/nicht-GV-Status der durch neue Techniken entwickelten Pflanzen. Die vorliegende Arbeit beinhaltet jedoch keine Einstufung der neuen Techniken bezüglich ihres GVO-Status.

Einige neue Techniken werden bereits vom Pflanzenzüchtungsektor in der Forschung und Entwicklung verwendet. Allerdings sind bisher noch keine Produkte, die durch die Anwendung neuer Techniken entwickelt wurden, am Markt erhältlich. Wie schnell solche Produkte vermarktet werden, hängt von vielen Faktoren ab, einschließlich der Entscheidungen der Gesetzgeber auf europäischer Ebene. Der vorliegende Bericht kann daher nur Hinweise bezüglich der zu erwartenden Entwicklungen und ihren praktischen Konsequenzen geben.

Im Rahmen der Risikobewertung der untersuchten Pflanzen ist eine gründliche molekulare Charakterisierung notwendig, welche beweist, dass nur die beabsichtigte Änderung stattgefunden hat, und dass weder unbeabsichtigte Effekte noch unerwünschte Insertionen vorliegen. Die Mindestanforderung ist daher eine molekulare Analyse und Dokumentation, welche die Sequenz des Zielbereichs und der flankierenden Sequenzen im Detail beschreibt. Dies, zusammen mit einer phänotypischen Analyse, stellt die Basis für weitere (Fall-zu-Fall-) Entscheidungen über die notwendigen Elemente in der Risikobewertung dar.

Die Methoden der Wahl, um DNA in Pflanzenzellen einzubringen, sind in den meisten Fällen analog zu denen der Transgenetik. Cisgenetische Pflanzen werden in der Regel durch Agrobakterien-vermittelte Transformation hergestellt. Diese Methode neigt dazu, Mutationen wie Deletionen und Umlagerungen innerhalb der genomischen DNA von Pflanzen zu verursachen. Es muss berücksichtigt werden, dass sich die rekombinante DNA Technologie von der meiotischen Rekombination unterscheidet. Unerwünschte Wirkungen, die aus dem Transformationsprozess resultieren, müssen auf ein möglichst geringes Ausmaß beschränkt werden.

Im Hinblick auf den verfügbaren Genpool werden nur Gene von sexuell kompatiblen Arten in cisgenetische Pflanzen übertragen, während bei der Mutagenese endogene Gene verändert werden. Daher können Elemente des Risikobewertungsverfahrens - wie durch Gentransfer verursachte Umweltrisiken - neu beurteilt werden. Die Tatsache, dass ein modifiziertes Gen und gegebenenfalls ein neu exprimiertes Protein aus der gleichen oder kompatiblen Art stammt oder direkt im Genom modifiziert wurde, senkt die Wahrscheinlichkeit, dass mögliche schädliche Auswirkungen auf die Umwelt auftreten. Wenn ein Gen bereits in der kompatiblen Population vorhanden war, sind eine Reihe jener Risiken, die mit transgenen Pflanzen assoziiert werden, vermutlich nicht relevant. Zusätzlich ist es möglich, dass die Anwendung der neuen Techniken in Pflanzen resultiert, die sich in ihren Eigenschaften nicht

wesentlich von traditionell gezüchteten unterscheiden. Daraus kann geschlossen werden, dass die mit ihnen verbundenen Risiken für die menschliche und tierische Gesundheit ähnlich sind, und die Beurteilung der Lebensmittel- und Futtermittelsicherheit kann entsprechend durchgeführt werden.

Zusammenfassend gilt, dass die Grundlage für die Beurteilung potenzieller Risiken von durch neue Techniken hergestellten Pflanzen und daraus hergestellter Lebensmittel und Futtermittel gleich jener für gentechnisch veränderte Pflanzen ist. So sind die jeweiligen EFSA Guidance Documents für die Beurteilung von Lebensmitteln und Futtermitteln sowie für die Durchführung der Umweltrisikobewertung anzuwenden. Abhängig von den Eigenschaften der untersuchten Pflanze können die Datenanforderungen von Fall zu Fall neu überprüft werden.

Durch neue Techniken entwickelte Pflanzen zeigen häufig Merkmale, die sich auf Labortests und die Nachweisbarkeit in Versorgungsketten auswirken. Unabhängig von der angewandten Technik ist es erforderlich die Stelle der genetischen Veränderung im Genom zu kennen. Mit diesen Informationen können grundsätzlich Nachweismethoden für alle Pflanzen, die mit Hilfe neuer Techniken hergestellt wurden, entwickelt werden. Cisgenetische Pflanzen beinhalten eine einzigartige Kombination der eingefügten und der flankierenden Sequenzen im Genom der Pflanze, was den Event-spezifischen, eindeutigen Nachweis und die Quantifizierung möglich macht. Einige genomische Veränderungen sind vermutlich nicht von denen, die durch traditionelle Pflanzenzüchtung entstehen, unterscheidbar. Zum Beispiel können Punktmutationen, die durch ODM oder ZFN-1 induziert sind, nachgewiesen werden, aber es ist nicht möglich den Ursprung der Mutation zu identifizieren. Wenn ZFN Typ-3 mit Cisgenetik kombiniert wird, um eine endogene Sequenz durch eine sehr ähnliche einer kompatiblen Art zu ersetzen, ist es wahrscheinlich schwierig, geeignete Nachweismethoden zu entwickeln.

Ein wichtiger Aspekt für die Kennzeichnung eines GVO Rohstoffs oder Erzeugnisses ist die Verfügbarkeit von zuverlässigen Protokollen für den eindeutigen Nachweis und die Quantifizierung. Schwellenwerte für das zufällige oder technisch nicht zu vermeidende Vorhandensein von zugelassenen GVO wurden festgelegt, und die Kennzeichnung von Produkten (Material), die aus GVO bestehen, solche enthalten oder daraus hergestellt wurden, basiert gegenwärtig auf der eindeutigen Quantifizierung des Vorhandenseins eines zugelassenen GVO. Es kann schwierig sein, das Vorhandensein von durch neue Techniken entwickeltem pflanzlichen Material zu quantifizieren und somit den Kennzeichnungsvorschriften gemäß den aktuellen GVO-Rechtsvorschriften zu folgen. Dies ist wahrscheinlich der Fall für ODM und ZFN-1, die zu

Punktmutationen führen, für die die Entwicklung einer eindeutigen Quantifizierungsmethode nicht einfach ist.

Im Falle eines autorisierten GVO wird das Vorhandensein üblicherweise durch geeignete Nachweismethoden nachvollzogen, die auch für Überwachungszwecke notwendig sind. Die Rückverfolgbarkeit basiert weitgehend auf Dokumentation, und allgemeine Systeme der Rückverfolgbarkeit sind in den großen Lieferketten für Lebensmittel, Futtermittel und Saatgut vorgesehen. Unabhängig vom GVO-Status kann die Anwendung einer neuen Technik einfach durch geeignete Dokumentation verfolgt werden, wenn der Antragsteller die entsprechende Information während der Sortenregistrierung zur Verfügung stellt. Für die Rückverfolgbarkeit von GVO sind eigene rechtliche Maßnahmen in Kraft; ihre Anwendbarkeit hängt davon ab, wie die Pflanzensorten klassifiziert werden (GV/nicht-GV Status), die durch neue Zuchtmethoden entwickelt wurden.

Bevor eine Pflanze, die ein GVO-Event beinhaltet, verwendet werden kann, um eine neue Sorte zu entwickeln, muss das Event von der Europäischen Kommission genehmigt werden. Darüber hinaus werden GV-Sorten in den Gemeinsamen Sortenkatalogen deutlich durch Fußnoten mit dem Hinweise auf die Zulassung eines Events mit der entsprechenden Kommissionsentscheidung gekennzeichnet. Zu Beginn der Sortenregistrierung muss der Antragsteller eine schriftliche Bestätigung beibringen, ob die Sorte GV ist oder nicht. In der Saatgutproduktion ist die allgemeine Rückverfolgbarkeit aufgrund der Anforderungen des Europäischen Saatgutzertifizierungssystems gegeben. Saatgutpartien werden zur Zertifizierung beantragt, und spezifische Identifikationsnummern und definierte Aufzeichnungspflichten sind während der gesamten Saatgutproduktion, den Verarbeitungs- und Verteilungsprozessen vorgesehen. Wenn Saatgut aus einer gentechnisch veränderten Sorte produziert wird, ist die eindeutige Kennzeichnung des Saatgutes als GV während der gesamten Saatgutproduktion obligatorisch. Lebens- und Futtermittel, die aus gentechnisch verändertem Saatgut hergestellt werden, unterliegen den nationalen und europäischen Rechtsrahmen für GVO.

Die Klassifizierung der resultierenden Pflanzen (GV/nicht-GV) wird als entscheidend im Hinblick auf die praktischen Auswirkungen der Anwendung der neuen Techniken in der Pflanzenzüchtung identifiziert. Der Status der Pflanzen bestimmt die anzuwendenden Maßnahmen der Gesetzgebung und folglich die Risikobewertung, den Nachweis und die Rückverfolgbarkeit.

1 Background

Plant breeding and genetic modification techniques have developed rapidly since the release of the first genetically modified (GM) plants.

A comprehensive body of legislation concerning GMOs has been established in the European Union, with the aim to ensure a high level of protection of human life and health, animal health and welfare, the environment and consumer interests. The regulatory framework, *inter alia*, foresees detailed provisions concerning traceability and labelling.

The present European Union (EU) legislation defines genetically modified organisms (GMOs) as follows:

For the purposes of Directive 2001/18/EC

(1) 'organism' means any biological entity capable of replication or of transferring genetic material;
(2) 'genetically modified organism (GMO)' means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination;
Within the terms of this definition:
(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1;
(b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification;

In some cases the modifications of the genome achieved through novel breeding technologies are expected to be indistinguishable from those occurring during traditional plant breeding. In this respect, the current definition of GMO in the EU legislation led to discussions concerning the GM/non-GM nature of the corresponding plants. It is not within the scope of this report to evaluate whether the existing regime is applicable to plants produced through novel techniques. The definition as GMO/non-GMO is, however, of utmost importance for practical consequences arising from the application of the new techniques.

In 2007 a working group on new techniques was established to analyse new biotechnological techniques at the request of the Competent Authorities under Directive 2001/18/EC; the scientific experts assessed whether the application of these techniques results in a GMO according to the current definitions and a corresponding report was published. In addition, the Joint Research Centre

(JRC), the Institute for Prospective Technological Studies (IPTS) and the Institute for Health and Consumer Protection (IHCP) published a joint report on the state-of-the art and prospects for commercial development concerning new plant breeding techniques (Lusser et al. 2011). In particular, cisgenic and intragenic plants – plants with genomes modified with genes from the species itself or from a crossable species - have been identified as techniques of high interest, in particular in Europe. At present, the Netherlands are the most active EU Member State in the development and promotion of cisgenic plants, reflected by a number of publications and reports, seconded by – also academic – working groups in Germany and Italy, as well as in the non-EU Member State Switzerland. As a follow up of the JRC report (Lusser et al. 2011) a workshop was held to compare regulatory approaches for new plant breeding techniques (Lusser and Rodríguez Cerezo 2012).

Notwithstanding their "conceptual diversification" (Nielsen 2003), transgenesis, intragenesis and cisgenesis use the same genetic modification techniques (Schouten and Jacobsen 2007). As opposed to transgenesis, in which DNA fragments from any organism may be inserted into a genome, cisgenesis and intragenesis are two new concepts proposed for the genetic modification of plants. Transgenesis may extent the gene pool of the recipient species whereas cisgenesis (Schouten et al. 2006) contains only genes from inside the sexually compatible gene pool. Cisgenic modifications are achieved with genes from the same or cross-compatible species (close relatives) including associated introns and regulatory elements in their natural state. By contrast, intragenesis (Nielsen 2003, Rommens 2004) allows for the utilisation of new gene combinations – but still from crossable species – created by *in vitro* rearrangement of functional genetic elements such as promoter regions, coding regions with or without introns and terminal regions. In cisgenesis, such rearrangements are not permitted and the "cisgene" is accordingly a complete copy of the endogenous gene including the promoter, introns and the terminator in the normal-sense orientation (Lusser et al. 2011).

1.1 Impact of new techniques and international developments

The relevance of the topic is at least in part due to the strong publicity of some stakeholders. However, as cisgenic and intragenic plants are expected to be more accepted by the consumers (see Eurobarometer, European Commission 2010), commercial R&D efforts will very likely result in their placing on the market within the next few years. Consequently, the European Commission (EC) launched a number of activities to clarify the consequences of breaking innovations in plant breeding. Already in 2007, the EC set up a Working Group on New Techniques ("new techniques working group") to evaluate the status of plants obtained through new biotechnological techniques as being GM or non-

GM according to the legal definition. In June 2011, a hearing on cisgenesis in plant breeding was held on the premises of the European Parliament. The European Food Safety Authority (EFSA) has prepared an evaluation of the adequacy of EFSA guidelines to perform a risk assessment of plants developed through cisgenesis (mandate EFSA-Q-2011-00152, EFSA 2012).

Cisgenesis

In the USA and New Zealand, research and development (R&D) has put some focus on both cisgenic and intragenic plants, and is primarily in the hands of companies. The United States Environmental Protection Agency (EPA) indicated to reduce the regulatory requirements for plant-incorporated protectants (PIPs). Through this, they hope to promote research and development but also to reduce the number of applications for registration (Waltz 2011, EPA 2011 www.epa.gov). The pending decision of the US Environmental Protection Agency to exempt cisgenic PIPs is discussed controversially, because it could imply a fundamental difference between transgenesis and cisgenesis although they use the same techniques for achieving the genetic modification.
In New Zealand, the major driving force for the development of cisgenic crops is the anticipated higher consumer acceptance.

ODM

Until 2011, 26 patents in the USA and EU have been submitted concerning ODM to induce mutations (Lusser et al. 2011). The relatively large number of patents indicates that the technique is clearly intended for commercial applications. In the United States, plants developed through ODM have been declared non-GM by USDA APHIS (U.S. Department of Agriculture, Animal and Plant Health Inspection Service; ISB 2009; Waltz 2012). An herbicide-tolerant canola produced through ODM could already be released this year (Waltz 2012).

ODM, in particular the patented Rapid Trait Development System (RTDS,) has been declared non-GM, or more precisely as mutagenesis technique, by the US Department of Agriculture (USDA; Breyer et al. 2009; Waltz 2012; European patent EP1223799). RTDS makes use of a chemically synthesized “gene repair oligonucleotide” (GRON) as a template. It comprises approximately 68 nucleotides, and includes a nick and a hairpin structure. Employing this approach, several co-operations within industry have been launched to develop crops that are marketed as non-transgenic. The partnerships, in which the RTDS technology is employed, should lead to the release of a number of crops with different traits, including herbicide tolerance and pest resistance. The crops are partially undisclosed and with different traits, including multitraits.

Chemically synthesized single-stranded DNA oligonucleotides are the method of choice in commercial applications (Waltz 2012). The first commercial product produced through ODM is herbicide resistance in rapeseed and canola, maize and rice. To achieve this, mutations of the genes *ALS* in rape and rice, or *AHAS* in maize have been induced. Herbicide tolerance is the most advanced (and soon to be commercialized) trait. Herbicide tolerant canola with resistance to imidazolinone herbicides is in registration trials, and it is expected that the trait will be commercialized in 2013. RTDS-bred, herbicide-tolerant oilseed flax may be on the market in 2015 (Flax Council of Canada 2012); other crops include potato, sorghum, soybean and wheat. Also traits different from herbicide tolerance are in the focus, e.g. the modification of vegetable oils. Research also focuses on the mutation of genes in potato to create resistance against *Phytophtora* ssp.

ZFN

There is a lot of interest about the potential applications of zinc finger nucleases (ZFNs), as these enzymes allow highly specific, targeted genome modification in live cells.

However, questions have been recently raised about the purported specificity of these genome modification tools. They show that ZFNs, in addition to cleaving at their desired sites, can also have unexpected cleavage effects *in vivo* that cannot be predicted using conventional *in silico* analyses (off-target). This can be brought about by insufficient specificity of DNA binding, hence allowing ZFN activity at similar target sequences within the genome, or by activation of the ZF nuclease domains before the nuclease is properly bound to the DNA. These findings could have important consequences for the safe use and optimisation of ZFNs (Cheng et al. 2011).

Moreover, Gupta et al. (2011) stated that, although ZFNs have been used to create genetically engineered organisms, the characterisation of ZFN-induced collateral damage to the genome of treated cells has been limited primarily to indirect assays of toxicity and DSB foci or lesion analysis at a small number of potential off-target sequences.

Internationally, plants modified through zinc-finger nucleases (ZFN) are at least case-by-case exempt from regulatory review, similar to ODM-modified crops. In Canada, crops with novel traits have to pass safety assessments and an authorisation process, independent of the technology used (i.e. traditional breeding, cell fusion, mutagenesis, recombinant DNA techniques etc.). In case of herbicide-tolerant crops, the trait, not the method, triggers legislation (Lusser and Rodríguez Cerezo 2012). In Australia, it seems likely that crops developed through the use of the ZFN-1 technique will not be regarded as GMOs (Lusser

and Rodríguez Cerezo 2012). The USDA will decide on ZFN case by case, which indicates that some products might be exempted from further review (Waltz 2012). However, this implies that companies will have to consult with the USDA before they can place products developed through ZFN on the market.

1.2 Different scenarios

The underlying rationales for international (pending) decisions concerning the regulation of plants derived through novel plant breeding techniques are largely not documented. In the European Union, different scenarios concerning such plants are deemed possible – to exempt crops produced using novel techniques from the current GMO regulatory framework, to reduce the requirements for their risk assessment, or to sustain the current risk assessment practices. In the present report, the impacts of two scenarios with a focus on cisgenesis, ODM and ZFN are discussed: Scenario A - full requirements for GM plants apply, and Scenario B - plants are not covered by the current regulatory requirements for GM plants. Furthermore, the practical consequences of the scenarios on risk assessment, detection and traceability, are highlighted. Possibilities to combine cisgenesis with other novel techniques under investigation (ODM, ZFN, Agroinfiltration) are highlighted.

2 Detailed definitions

2.1 Transgenesis and intragenesis

Nielsen (2003) suggested categorising genetically modified – or transgenic – organisms (*i.e.* organisms changed by receiving hereditary material from another organism) according to the origin of the genetic material used for the modification. Based on the "genetic relatedness between the donor and the recipient organisms", five categories of GMOs were presented, including a definition of "intragenesis". In this classification "intragenic organisms" would contain genes from within the genome, they could derive from traditional breeding, and the genetic distance between donor and recipient would be low. At the far end of the spectrum "xenogenic" organisms would be characterised by high genetic distance due to completely designed genes, and modifications impossible to achieve through traditional methods.

Rommens (2004) – in accordance with the classification proposed by Nielsen (2003) – used the term "**intragenic**" to describe plants harbouring genomic material from crossable species ("the same sexual compatibility group"). Genomic elements from sexually compatible relatives could be arranged freely. Concurrently, the same author described the concept of "**all-native DNA transformation**" (Rommens 2004), and proposed that adequate plant sequences could replace foreign regulatory elements, such as bacterial terminators.

In the intragenic method, genetic elements are isolated from plants, rearranged *in vitro* and introduced into plants from within the sexual compatibility group (Rommens et al. 2007). The authors claim that intragenic crops are comparable to those developed through traditional methods as no traits that are new to the sexual compatibility group are introduced. Consequently, intragenic plants do not contain foreign genes such as selectable marker genes or insecticidal genes. Also gene silencing techniques may be employed, e.g. through RNAi (Lusser et al. 2011).

Rommens (2004) also reported on the production of marker- and backbone-free potato plants following the intragenic concept. In addition, the discovery of plant transfer DNAs (P-DNAs) as well as plant-based selection systems rendered the exclusive use of plant genes for transformation purposes possible. The new technical possibilities led to the perception that intragenic vectors contain functional equivalents of the usual, non-plant vector components, a concept presented by Rommens et al. (2004). Ideally, they should originate from the

same or a crossable species (*i.e.* the gene pool available to traditional plant breeders to date). The group subsequently described the identification, isolation and use of T-DNA border-like sequences within the genomes of a number of different plants like potato, tomato, pepper, rice, barley, maize and wheat (Rommens et al. 2005).

2.2 Cisgenesis

The term cisgenesis was coined by Schouten and colleagues (Schouten et al. 2006). They claimed that – despite using the same genetic modification techniques as in transgenesis – "cisgenic" plants could be compared to traditionally bred plants as the concept involves only genes from the plant itself or from a close relative. These genes could also be transferred by traditional breeding methods. The genomic region containing the gene of interest is left contiguous, including all regulatory elements.

"Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable – sexually compatible – organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation.
Cisgenic plants can harbour one or more cisgenes, but they do not contain any parts of transgenes or inserted foreign sequences. To produce cisgenic plants any suitable technique used for production of transgenic organisms may be used. Genes must be isolated, cloned or synthesized and transferred back into a recipient where stably integrated and expressed.
Sometimes the term cisgenesis is also used to describe an *Agrobacterium*-mediated transfer of a gene from a crossable – sexually compatible – plant where T-DNA borders may remain in the resulting organism after transformation" (cisgenesis with T-DNA borders (EFSA 2012; Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis).

The definition of cisgenesis in the EFSA scientific opinion refers to the definition of cisgenesis as coined by Schouten and colleagues (Schouten et al. 2006).

2.2.1 Common features of intragenesis and cisgenesis

Both concepts imply that plants are transformed only with their own genetic materials or genetic materials from closely related species capable of sexual hybridisation. Furthermore, foreign genes such as selection marker genes and vector-backbone genes should be absent or eliminated from the primary transformants or their progeny.

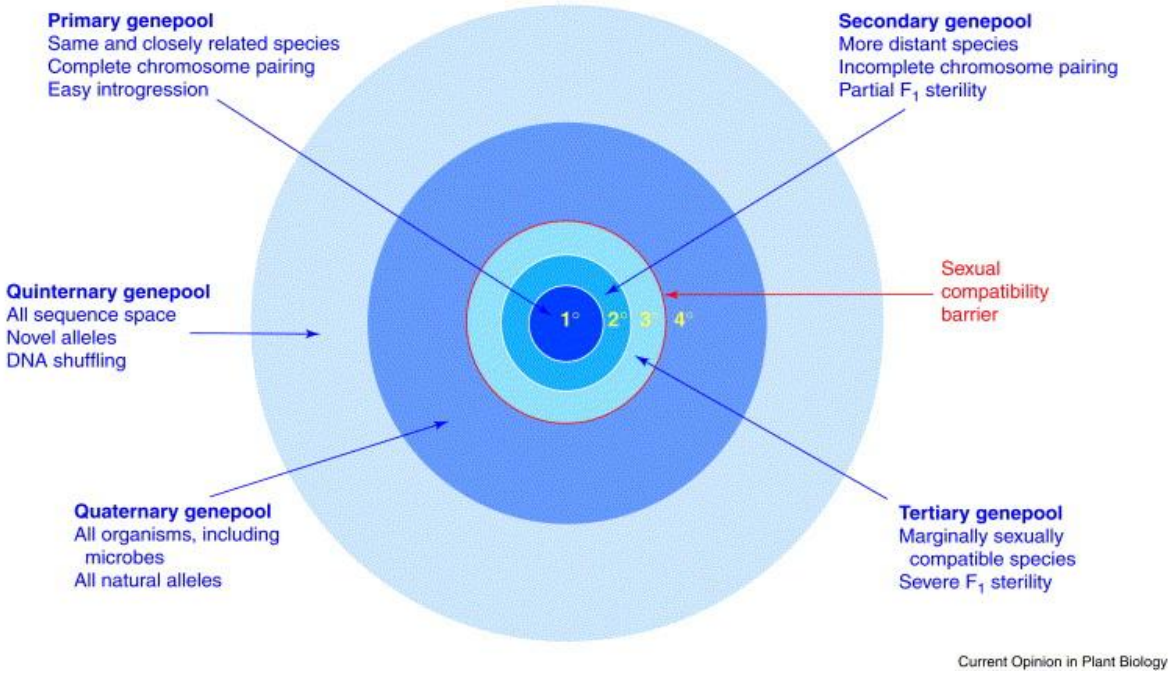


Figure 1. Different genepools for plant improvement, taken from Michelmore (2003)

The major features of transgenesis, intragenesis and cisgenesis – an overview

Transgenesis and cisgenesis use the same genetic modification techniques (Schouten and Jacobsen 2006). Transgenesis may extent the gene pool of the recipient species whereas intragenesis and cisgenesis contain only genes from inside the sexually compatible gene pool, *i.e.* from the plant itself or from a close relative (Figure 1). Transgenesis frequently creates completely artificial traits, and the source of DNA may be any species. Intragenesis allows designing traits using genetic elements from the crop itself that are rearranged *in vitro* (Rommens et al. 2007). Thus, also gene silencing approaches may be used. In contrast, according to the definition of cisgenesis no alteration whatsoever of the native status of a plant gene is permitted (Table 1).

Table 1. Major characteristics of different GM concepts

Transgene	• gene from outside the sexual compatibility group • could be from any organism • may contain marker genes of any origin for selection
Intragene	• gene, regulatory elements and other components from the plant itself or from crossable species • elements may be rearranged • silencing approaches possible • use of plant-derived sequences for gene transfer (P-DNA) *via Agrobacterium* • selection markers are removed
Cisgene	• contiguous gene from the plant itself or from crossable species • gene with all native components incl. promoter, introns and terminator regions • use of *Agrobacterium* sequences for gene transfer (T-DNA) • selection markers are removed

3 The EU regulatory definition of GMO

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

Recital 17 of Directive 2001/18/EC
clearly states that this Directive "should not apply to organisms obtained through certain techniques of genetic modification which have conventionally been used in a number of applications and have a long safety record."

Article 2 (Definitions)
"For the purposes of this Directive:
(1) 'organism' means any biological entity capable of replication or of transferring genetic material;
(2) 'genetically modified organism (GMO)' means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination;

Within the terms of this definition:
(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1;
(b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification;"

Annex I A (techniques referred to in Article 2(2), part 1
Techniques of genetic modification referred to in Article 2(2)(a) are inter alia:
(1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation;
(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;
(3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

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

(1) in vitro fertilisation,
(2) natural processes such as: conjugation, transduction, transformation,
(3) polyploidy induction.

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

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

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed
refers to Directive 2001/18/EC in its Article 2(5) to define GMOs (see "Definitions", Regulation (EC) No 1829/2003).

Article 2 (Definitions)
4. the definitions of 'organism', 'deliberate release' and 'environmental risk assessment' referred to in Directive 2001/18/EC shall apply;
5. 'genetically modified organism' or 'GMO' means a genetically modified organism as defined in Article 2(2) of Directive 2001/18/EC, excluding organisms obtained through the techniques of genetic modification listed in Annex I B to Directive 2001/18/EC;
6. 'genetically modified food' means food containing, consisting of or produced from GMOs;

4 Cisgenesis

4.1 Scientific peer-reviewed literature reporting experimental data

The in-depth analysis of the current scientific peer-reviewed literature claiming to present "cisgenic" or "intragenic" approaches shows that the approaches published as "cisgenic" do not necessarily satisfy the definition of cisgenesis *sensu stricto*. Currently only two articles – Vanblaere et al. (2011) and Holme et al. (2011) – are likely to fit the definition of cisgenesis as coined by Schouten et al. (2006). The published data strongly suggest that – using the currently available transformation methods – it is not possible to produce cisgenic plants containing only the cisgene without any further modifications.

Cisgenic Apple apple lines cv. "Gala" were produced by Vanblaere et al. (2011). They employed the ORF of the HcrVf2 genomic region from the wild relative *Malus floribunda*, including 242-bp from its 5' UTR and 220-bp from its 3' UTR and conferring scab resistance. The segment between the recombination sites that contains the *npt*II gene for kanamycin selection was removed through dexamethasone-induced recombination and thus resulted in marker-free lines. Presence of *HcrVf2*, absence of *trfA* (responsible for initiation of replication) and *npt*III as part of the backbone, and the fusion marker gene *npt*II/*codA* was demonstrated by PCR.

"Cisgenic barley with improved phytase activity" was demonstrated by Holme et al. (2011). They achieved the marker-free status of the cisgenic plants by using the pClean dual binary vector system that uses hygromycin resistance for selection (Thole et al. 2007). The genomic region of the *PAPhy_a* gene comprised 5208-bp and was amplified by PCR.

Kamrani et al. (2011) published a paper called "Cisgenic inhibition of the potato cold induced phosphorylase L gene expression and decrease in sugar contents". However, in their approach they used an RNA silencing construct, controlled by the 35S promoter and the OCS terminator, and selected putative transgenic shoots on kanamycin-containing medium. Removal of the selection marker was not reported.

Lütken et al. (2011) described an approach towards cisgenic modification of *Kalanchoë* that would replace the application of growth regulators. They state that the chemicals are potentially harmful to human health and the environment and thus will be banned in the EU in the near future. For this, they identified *KNOX* genes involved in vegetative vivipary and overexpressed two of

them (*KxhKN4* and *KxhKN5*) by introducing the complete cDNAs under the control of the 35S promoter and the NOS terminator. They also used a post-transcriptional gene silencing (PTGS) construct that contained a 326-bp fragment of *KxhKN5*.

Han et al. (2010) showed the insertion of five genes that encode proteins involved in gibberellin metabolism or signalling. All "cisgenes" were isolated and transformed along with their promoter and terminator regions (in this case 1-2-kb of 5' and 1-kb of 3' flanking DNA), and as contiguous sequences including all exons and introns. Basta (glufosinate-ammonium) was used for selection during plant regeneration (*bar* gene with NOS promoter and terminator), no removal of the selectable marker gene was demonstrated. The genes used in the study were expressed in the xylem and phloem and identified by microarray expression data. They observed a great variation in the large number of independent events they analysed. The successful insertion of the cisgene was PCR verified using primers directed at flanking T-DNA sequence that was not present in wild type plants.

An intragenic ("all-native DNA") approach was used by Rommens et al. (2008) to accomplish silencing of two asparagine synthetase genes in potatoes, a metabolic change finally resulting in low-acrylamide French fries and potato chips. They used potato-derived border-like elements instead of the widely used T-DNA borders, and two potato promoters (Gbss, Agpase). The presence of the gene silencing construct und absence of marker and backbone sequences were shown by PCR-based genotyping of the transformants. The *npt*III gene from *E. coli*, conferring kanamycin resistance, resides on the backbone of the vector and is usually removed during the regeneration process. Plant selection is based on the transient production of the natural cytokinin isopentenyl adenosine (Richael et al. 2008).

A detailed abstract was published by Kichey et al. (2009). The authors reported the production of barley with improved nitrogen use efficiency (NUE). Their cisgenic approach used the genomic sequence of *TIP2* (3532-bp), including promoter (1999-bp upstream) and terminator (564-bp downstream), and the *GS1* gene (*GS1a* isoform) which consisted of a 5.2-kb gene fragment, including 1.5-kb promoter and 491-bp terminator.

Kuhl et al. (2007) presented "a partially cisgenic event" in potato, which was achieved by introducing an 8.59-kb fragment of the *RB* gene conferring late blight resistance (including 2.5-kb upstream of the strt ATG and 2.48-kb downstream of the stop codon). As the selectable marker *npt*II was retained in the transformants they referred to them, by definition correctly, as "transgenic".

4.2 Anticipated developments using cisgenesis

4.2.1 Application of cisgenesis in plant breeding

The improvement of quality traits in plants is a major goal in plant breeding programmes, indicated also by trends in the pipelines of biotech-companies that currently employ transgenic methods. The targeted traits include fatty acid composition (omega-3 fatty acids, reduced saturated and increased unsaturated fatty acids contents, elimination of trans fats), enhanced flavour, fiber quality, improved shelf life, but also optimization for the use as food, feed, biofuel or industrial uses (see also Dunwell 2010; Stein and Rodríguez-Cerezo 2010).

This may also be seen as a response of companies to the limited consumer benefits of GM plants currently on the market, primarily aiming at cost reduction in the production process. Future plant breeding efforts, including transgenic approaches, will focus on breeding varieties with enhanced consumer traits having a direct advantage for the consumer, including functional, healthy and tasty foods (Kok et al. 2008). Quality traits (e.g. the accumulation of beneficial nutrients) are usually influenced by a plant's metabolic network, and thus frequently governed by enzymes. The manipulation of key enzymes may be used to achieve a desired effect. This may, depending on the activity of the promoter, lead to major alterations in a plant's general metabolism. Particularly when the plant's metabolism needs to be targeted by GM, unforeseen effects potentially occur due to various interactions within the metabolic network.

Introgression of desired genes, in particular single genes, may clearly be speeded up by cisgenic approaches. Hence, the most significant contribution of cisgenesis may be expected for the improvement of monogenic resistance traits. Major advantages could be expected in breeding of plants with long lifespans such as trees. Traits such as abiotic stress tolerance are usually complex (e.g. due to polygenic traits). The introgression of one gene or QTL is usually not sufficient to engineer stress-tolerant lines (Varshney et al. 2011). Gene pyramiding will be necessary in most cases, implying that the sequences and functions of genes are well characterised. Thus, cisgenesis is not expected to play a significant role in the improvement of stress tolerance.

Gene silencing cannot be attempted in a targeted manner with cisgenic methods. The cisgene by definition has to be inserted in sense orientation and unchanged, thus neither antisense technologies nor RNA interference (RNAi) are possible. Gene silencing could, however, be achieved using intragenesis. Strong and – if appropriate – ubiquitous plant-derived promoters, may be used for such purposes. Plant actin or ubiquitin promoters have been widely used in plant research and are thus well-characterised. However, intragenesis will be the

method of choice to achieve gene silencing. Accidental gene silencing could occur in rare cases, based on the same mechanisms like in transgenic plants.

Currently it is hard to evaluate how quickly companies would be able to bring cisgenic crops to the market. Their marketing policy may in the end also depend on decisions concerning the regulatory framework.

4.2.2 Bottlenecks for the practical use of cisgenesis

- The major bottleneck may be the identification of genes encoding for the desired traits. Genes need to be fully characterised, including potential interactions.

- Many important traits in plants constituting major breeding goals result from the interaction of several genes.

- To ensure that new resistances are not broken rapidly, a combination of genes should be inserted into the recipient plant (gene stacking, multigene cassettes).

- Isolated genes and their regulatory elements are introduced into a different genetic background. It has to be proven whether they retain their anticipated function in an altered genetic background.

- Plant transformation remains a tedious procedure, in particular in fruit trees (see Petri et al. 2009). The same limitations (e.g. random integration of genetic constructs) as for transgenic plants apply.

- Current plant transformation methods rarely lead to the desired results and the selection of an appropriate plant is costly as many plants have to be thoroughly analysed. Cisgenic plants need to be described in detail, as their characteristics are clearly defined.

- The complete removal of selection markers is, by definition of cisgenesis, indispensable; the efficiency depends on the method applied and needs to be verified individually.

- Native promoters may lead to constitutive expression of genes, which may be above the native expression level of a gene, as shown for the *HcrVf2* gene conferring apple scab resistance (Szankowski et al. 2009). The altered expression can alter the environmental behaviour of the plant and also render considerations concerning exposure of potential consumers necessary.

- Following the definition of cisgenesis, gene silencing is not possible.

Cisgenesis has been tested in the Netherlands, Germany, Switzerland and Italy, in particular in fruit trees. However, also forest trees (e.g. poplars) that are used for wood or energy production are improved through cisgenic approaches.

Stress tolerance and disease and pest resistance (plant incorporated protection, PIP) are currently major goals of plant breeders and researchers working on the development of cisgenic crops. Also quality aspects may be improved by incorporating additional copies of a given gene.

In order to use cisgenic approaches for crop improvements, genes associated with the desired trait must be defined. Molecular markers may assist their identification, especially as they have become important tools of traditional plant breeding methods (Collard and Mackill 2008). For biotech-approaches, genes of interest must be isolated. The identification and isolation of these genes are greatly facilitated by continuous achievements in plant genome sequencing. Steadily updated databases are useful tools for *in silico* research. In addition, the publication of complete sequences of more and more crops, including apple and potato (Pennisi 2011; see Velasco et al. 2010 for apple; The Potato Genome Sequencing Consortium 2011 for potato), opens up new horizons of genomics research. Consequently, at least theoretically, the number of genes available for cisgenic or intragenic modifications is increasing. Their association with specific functions may be based on sequence similarities. The approach to identify sequences of putatively similar function through database searches has been exploited for the identification of plant-derived DNAs (P-DNAs, used as substitutes for conventional T-DNAs in *Agrobacterium*-mediated transformation) in a number of plant genomes (Rommens et al. 2005). Nevertheless, an important prerequisite for the efficient use of identified genes is their detailed experimental characterisation, which is cost- and time-consuming.

4.2.3 Cisgenic apples

The relatively long duration of tree breeding, which may last decades using traditional methods, makes the genetic modification of trees an attractive target (Harfouche et al. 2011). Traditional breeding of apple – and of tree breeding in general – is a tedious procedure caused by self-incompatibility, linkage drag, and the long time needed to develop a new cultivar. Other constraints, besides protracted generation cycles due to long juvenile phases, are the requirement of substantial space for the planting of seedling populations in the field, which renders tree breeding a labour- and cost-

intensive process. Like in all other modern breeding approaches, marker-assisted selection may greatly facilitate the identification of resistant individuals, also in breeding involving genetic modifications (Flachowsky et al. 2011).

Apple monocultures require the intensive application of plant protection products. Disease resistant varieties may lead to obvious consumer and environmental benefits, as less plant protection products are used. Additional benefits would be the reduction of disease control costs, and minimised pesticide residues on products from disease resistant cultivars (Penrose 1995; Kühn and Thybo 2001). Both apple scab, caused by the fungal pathogen *Venturia inaequalis*, and fireblight, caused by the bacterial pathogen *Erwinia amylovora*, are highly destructive diseases. In integrated approaches to control these diseases, horticultural practices play an important role. They are of utmost importance when the use of chemical measures is limited (Ozrenk et al. 2011).

In any case, there is clear necessity for fireblight-resistant cultivars of fruit and ornamental trees, as the disease is currently controlled with an antibiotic (streptomycin; Schlangen et al. 2007). Fireblight was believed to be endemic for North America, but has, however, spread across Central Europe and was detected in Austria for the first time in 1993 (Mayer et al. 2011).

The development of new apple cultivars by introgressing resistance genes into elite apple germplasm is a time-consuming and laborious process that may be accelerated by marker-assisted selection. Still, the introgression of disease resistance genes by traditional methods may not lead to commercially viable cultivars (Belfanti et al. 2004). Because of the possibility to transfer genes of interest into elite cultivars without impairing their characteristics, biotechnology has gained much attention for the development of disease resistant varieties.

Already decades ago, numerous resistance breeding programmes have been initiated to control apple scab. Apart from tedious breeding procedures, scab-resistant cultivars were sometimes described to be of low sensory quality, they may have a tough skin, the resistance may be negatively correlated to apple flavour, and storability may be limited (Kühn and Thybo 2001; Korba et al. 2008 and references therein). Using biotechnological approaches commercial varieties may be modified in a targeted manner, leaving their favourable quality characteristics unchanged.

Monogenic resistance and the identification of the corresponding resistance gene, e.g. in some wild *Malus* species (Vinatzer et al. 2001), is an ideal candidate for engineering resistance through biotechnological methods. Important sources of resistance are the *Vf* genes (due to their homology to the tomato *Cf*

resistance gene family named *HcrVf*, *i.e.* homologues to *Cladosporium fulvum* resistance genes in the *Vf*-region). The *HcrVf2* gene from the wild relative *Malus floribunda* 821 has been shown to induce scab resistance (Szankowski et al. 2009). The HcrVf locus and in particular the *HcrVf2* gene have been studied extensively in the past, and the Vf apple scab resistance was shown to co-segregate with other genes (Vinatzer et al. 2001; Szankowsi et al. 2009). In case of traditional breeding, such co-segregation may be either detrimental or beneficial, depending on the structure of the locus. If other, possibly interacting resistance genes are co-inherited with the gene of interest, the resistance may not be overcome so easily. Monogenic resistance is prone to resistance breakdown, as is the case for the widely used *HcrVf2*, which was overcome by some strains of the pathogen. This shows that the durability of the resistance induced by cisgenic methods may easily be threatened if based on single genes.

Nevertheless, despite many different approaches to manage pests in apple orchards (Figure 2), disease resistance remains a highly pursued and strongly researched trait in apple breeding (Cooley and Autio 1997; MacHardy 2000; Ozrenk et al. 2011). Integrated pest management systems could also assist prolonged disease resistance in GM varieties.

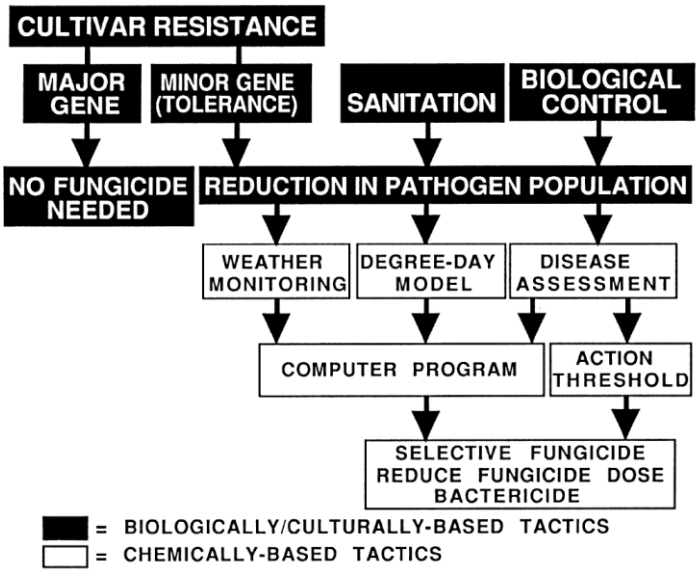


Figure 2. Disease management in apple orchards (MacHardy 2000)

Gene pyramiding (the combination of multiple – in particular resistance – genes into a single genotype) and the use of alternative genes may contribute to accomplishing durable resistance. Soriano et al. (2009) listed eleven major apple scab resistance genes mapped, allowing plant breeders to exploit them for the development of resistant apples. The pyramiding of several genes could be accomplished with traditional or biotechnological methods; however, gene interactions might differ depending on the genetic background into which they

have been introduced. In this respect, due to the more targeted insertion of introgressed genes, traditional methods may be advantageous.

A number of European institutes are currently working on cisgenic/intragenic apples (including Swiss Federal Institute of Technology (ETH) Zürich, Switzerland, Plant Research International Wageningen, the Netherlands, or the Julius Kühn Institute (JKI), Institute for Breeding Research on Horticultural and Fruit Crops, Dresden-Pillnitz, Germany). Besides European researchers, also plant breeders in New Zealand are active in applied research related to the use of plant genes for variety improvement.

Like for transgenic approaches (see Gessler and Patocchi 2007 for a review), the main breeding goals are the resistance against apple scab and fireblight. As mentioned above, due to – to some extent – monogenic disease resistance the trait could be engineered by introducing single genes. Moreover, the traits could be taken from wild relatives or from various cultivars, as also commercially successful cultivars show different degrees of disease resistance. The associated genes and loci have extensively been used also in traditional fruit tree breeding.

Cisgenesis could be employed for the quick introduction of desired traits into commercially successful cultivars without changing their favourable characteristics through introgression by traditional methods. In general, gene transfer technologies may effectively shorten the juvenile phase of fruit trees (Flachowsky et al. 2011).

Besides disease resistance, also better handling properties (e.g. for storage and transport) are of interest to R&D. Such traits would primarily be engineered by intragenic approaches, as frequently enzymes cause plant products to deteriorate, and gene silencing approaches like RNAi could be used to inactivate them.

4.2.4 Cisgenic potato

Potato breeding has a very long tradition. The life cycle of potato varieties is – compared with other field crops - rather long. Bintje and Russet Burbank for example, which are still significant on our potato markets, have been bred more than 100 years ago.
But the business has changed and the breeding objectives as well. Multi-purpose varieties for example are continuously replaced by more specifically ones.
And the breeding tools and methodologies have changed as well with the huge increase in scientific knowledge in nearly all relevant fields.

The list of potential breeding targets in potatoes is longer than in most other crops and depends on many different factors. Some of them are:
designated use (e.g. table, starch, crisps, french fries) designated cultivation area (e.g. temperature, precipitation, humidity, latitude, altitude) shift in production conditions (e.g. global warming, new species or strains of pests and diseases) other economic and market influences restrictions in production (e.g. organic farming) consumer attitudes (e.g. colour of skin and flesh, size of marketable tubers).

One example for the shift in breeding targets is the fact that marketing of table potatoes has changed nearly completely to washed products. As a consequence, the importance of surface quality has increased considerably.

Among the available breeding tools, cisgenetic methods gain increasing importance. Below, there are examples for the actual and potential use of cisgenetic tools in potato breeding activities.

Disease resistance

Potato suffers from many pests and diseases. Among them late blight, caused by the fungal pathogen *Phytophthora infestans*, is the one with the highest damage potential world-wide. As a consequence breeding efforts are enormous in order to get less susceptible and resistant new varieties, respectively, and new technologies are used especially in this breeding sector. The available sources for resistance genes are very large. Approximately 200 wild *Solanum* species with potential resistance genes are known in Middle and South America. Only a small percentage of them have been explored for use in breeding programmes up to now (Jansky 2006).

Classical breeding methods were not very successful during the last decades. At least two resistant Dutch varieties could be listed in 2005, 46 years after beginning of the breeding efforts. The resistance in those two varieties is based on single genes. So the probability of a resistance breakdown due to adaption of the pathogen is very high (Haverkort et al. 2009).

In a Dutch project called DURPh (Durable Resistance against *Phytophthora*), which has been started in 2006 under substantial public support, cisgenic breeding tools are used in order to get up to four different resistance genes into one variety without changing other original traits of the modified variety (Haverkort et al. 2009). In this way it should be possible that multiple *R* genes can contribute to a more durable resistance against late blight (Zhu et al. 2011). The availability of resistant varieties would lead to enormous reduction of the costs for plant protection measures as well as of the losses of yield.

Another breeding effort, where cisgenic strategies may be promising, is the resistance to nematodes and warts. In order to enhance quality traits, gene silencing is a tool to influence starch composition, processing quality and storage characteristics (Jacobsen and Schouten 2008).

Quality traits

Kamrani et al. (2011) reported of breeding successes in reducing the sugar content of two varieties significantly by gene silencing using “cisgenic measures”. However, their work does not conform with the definitions of neither cisgenesis nor intragenesis. Storage of potatoes at low temperatures has advantages like natural control of sprout growth, easier maintenance of the high humidity atmosphere required to minimise transpirational losses and reductions in senescent sweetening and losses due to storage rot. But a cold induced enzyme causes degradation of starch and increase of sugar content (reducing and non-reducing sugars). The reducing sugars glucose and fructose, participate in the Maillard reaction with free amino acids during frying resulting in dark-brown-coloured fries and chips. These darkened chips and fries are unacceptable to consumers and also may result in greater amounts of acrylamide production which has been linked to many cancers (Kamrani 2011).

In the media, the acrylamide-free potato "Ranger Russet" developed by an US-based company has been promoted as being cisgenic and appeared in several media reports. However, detailed analysis of the related publication (Rommens et al. 2008) shows that it is not appropriate to refer to this potato as “cisgenic” but rather “intragenic”. This is an example that, unfortunately, in particular in popular scientific literature and in media reports the term “cisgenic” is not used in an appropriate manner and frequently confused with “intragenic” modification.

The same company is also very active in the development of marker-free plants (diverse patents) and is the major proponent of intragenic modifications. Acrylamide-free potato "Russet Boise" is also part of the company’s portfolio.

An overview of genes and traits including their sources is presented in the annex (part 3).

4.2.5 Literature research: database searches and overview

Search engine based scans for novel plant techniques resulted in a plethora of scientific documents, theoretical papers, websites and further links. More than 300 citations concerning cisgenesis and intragenesis were derived from the web searches. They were primarily categorized as scientific and informative.

Cisgenic, intragenic as well as other novel plant breeding approaches are mentioned and discussed in several review articles and theoretical papers. Experimental papers describing the experimental approach thoroughly are scarce; scientific information is mostly published in conference proceedings or as presentations, providing only superficial information.

ODM is known under a number of different names, and the list according to Lusser et al. (2011) has been taken into consideration.

The following presents a non-exhaustive list of terms used in the website searches:
all-native plant transformation, P-DNA, cisgenesis, intragenesis, cisgenic plant(s), cisgenic, novel breeding techniques, cisgenic GMO, marker free transformation, GMO-detection/traceability, plant breeding, mutagenesis, mutation, disease resistance, apple scab, fireblight, late blight, herbicide resistance, ODM, oligo(nucleotide), ZFN, zinc-finger nuclease, mutation, site-directed, agroinfiltration, cross-breeding, selection, single nucleotide, gene targeting, T-DNA, marker excision, marker-free, introgression, linkage drag, backcross (breeding), molecular pharming/farming, molecular marker, etc.
Where available, the full text of scientific articles describing cisgenic approaches was thoroughly assessed. Most citations concerning advanced product development concern fruit trees, in particular apple, and potato. Two scientific articles describe the development of cisgenic apple lines that, according to the given information, comply with the definition of cisgenesis. To date, no adequate article on potato has been published.
The particular changes in the plant genome that is expected resulting from the application of novel techniques are clearly defined. Additional unintended modifications like the insertion of superfluous sequences are not permitted. Notably, the characteristics of cisgenic plants are explicitly described.

The most active countries in the field of cisgenesis and intragenesis are the Netherlands and the USA, followed by stakeholders in New Zealand. Research in New Zealand focuses on fodder crops, in particular perennial grasses.

(Fruit) trees (Rosaceae) and vegetatively propagated crops like potatoes are currently the primary target for cisgenic modification. The possibility to develop a marketable product depends, inter alia, on the trait of interest (monogenic, oligogenic) and the availability of the gene (or several genes) responsible for its manifestation. In a first step, monogenic traits may be targeted. However, also gene pyramiding is feasible.

Trees, in general, are an attractive target for cisgenic modifications. The major reason may be seen in the decreased time needed for the development of a new cultivar that will be successful on the market (see Flachowsky et al. 2011).

In vegetatively propagated trees and vines, including fruits and nuts that employ highly heterozygous varieties and long generation times, backcrossing to transfer an engineered trait is effectively impossible. Transformation of existing varieties adapted to local climatic conditions and market preference may thus be a promising approach. One of the major advantages of direct gene transfer into an existing variety is that the characteristics important for the consumer (e.g. taste, appearance) remain unchanged. This is particularly important in products consumed directly as is the case for apples or potatoes. In fruit trees, the major breeding goals amenable to cisgenesis are resistance against important diseases and pests in fruit production (e.g. apple scab and fireblight cedar apple rust in apple, resistance against root-knot nematodes in peach). Similarly, potato disease resistance (primarily against late blight) is the primary goal of potato breeders for which the responsible genes are characterised. Also quality traits may be manipulated, like the induction of anthocyanin accumulation with the aim to achieve attractive fruit colour in red apples. Cisgenesis has also been recognised as a potentially useful strategy to enhance the biomass of trees suitable for bioenergy production (Harfouche et al. 2011). An example is the attempt towards cisgenic modification of the gibberelic acid pathway in poplar (ISB 2009).

“Cisgenic” is a registered trademark of a New Zealand-based company, who have adopted this method to engineer pasture species/fodder crops (e.g. ryegrass and clover). The company defines its approach as intermediate between cisgenesis and intragenesis, e.g. allowing the omission of introns in sequences to be introduced. In analogy to the intragenesis approach, they may use P-DNA. The R&D focuses on drought tolerant perennial ryegrass cultivars and such with increased biomass. Sustainability (e.g. pest resistance) may also be a breeding goal.

Besides cisgenic barley (Holme et al. 2011), the same Danish group has been working on cisgenic wheat for feed purposes. To date, cisgenic barley harbouring the purple acid phosphatase (*PAP*) gene has been published. The researchers aim at the improvement of phosphate availability, amino acid composition, the bioavailability of minerals, and digestibility (for instance cell wall and starch). Genes involved in the relevant pathways are, e.g. glutamine synthetase (*GS1a*), tonoplast intrinsic proteins (*TIP2*), purple acid phosphatase (*PAP*), and multiple inositol polyphosphate phosphatases (*MINPPs*). Their approach is expected to result in an environmental benefit, as reduced release of N and P is anticipated when feeding the cisgenically modified plants.

In strawberries, cisgenic disease resistance against *Botrytis cinerea* was approached by Schaart (2004) using the endogenous strawberry gene encoding for polygalacturonase inhibiting protein *PGIP*, observing the strict use of

strawberry-own DNA sequences as target gene and as promoter and applying a selectable marker removal method for the elimination of marker genes.
In conclusion, the searches revealed that despite the cisgenic concept is strongly propagated only few concrete examples that comply with the clear definition of cisgenesis have been published. In most cases no evidence is provided that the molecular characteristics of a plant indeed follow the cisgenic concept. The few experimental papers and academic work published to date that provide detailed information indicate that with current methods the cisgenic approach per definition is not implemented easily.

Table 2. Information on cisgenic/intragenic plants is of different elaborateness.

Species	Trait	Gene	Approach	Source	Country	Institution	Reference
Apple	fire blight resistance	SB-37			SUI	ETH Zürich	No
Apple	fire blight resistance	*shiva-1*			SUI	ETH Zürich	No
A. thaliana	resistance against fungal and bacterial pathogens	*NPR1*	over-expression		SUI	ETH Zürich	No
Apple	vector	*pMF1*			HOL	Wageningen UR Plant Breeding	Yes Schaart et al. 2004
Rye-grass	drought tolerance	*Lpvp1*		*Lolium perenne*	NZL	Pastoral Genomics	
Apple	induce anthocyanin accumulation/ red apple fruit colour	MdMYB 10	over-expression	*Malus domestica*	NZL		Yes Espley et al. 2007

5 Targeted (site-directed) mutagenesis and gene insertion

An alternative to screening for randomly mutated alleles derived from classical mutagenesis techniques is the use of gene-specific (targeted, site-directed) mutagenesis, effected e.g. through oligonucleotides, zinc-finger nucleases, or meganucleases. So far, gene-specific mutagenesis has been restricted almost exclusively to genes conferring selectable phenotypes. However, other possible and identified targets include modified oil and fatty acid content (cottonseed, sunflower, rapeseed, linseed, peanut, soybean, maize, rice, etc.), starch, and protein quality (amino acid content), enhanced uptake of specific metals (soil contamination with heavy metals), deeper rooting system, abiotic stress tolerance (drought, soil salinity, extreme temperature) or the resistance to diseases and pests (Kmiec et al. 2003). Furthermore, the production of mutants for analytical purposes may be an aim. In particular, genes involved in pathogen defence frequently display rapid evolution through mutation (Niewenhuizen et al. 2012). Consequently, site-directed mutagenesis may be employed to restore activities of, e.g. enzymes (Niewenhuizen et al. 2012), similar to the restoration of an inactive GFP transgene (Beetham et al. 1999).

Breeders would generally appreciate the possibility to introduce, in a targeted manner, genes conferring desired traits. It is believed that – due to the targeted alteration in the plant genome – new techniques like oligo-directed mutagenesis (ODM) or zinc-finger nucleases (ZFN) result in fewer unintentional changes/effects than observed in organisms generated by breeding techniques based on irradiation or chemical mutagenesis. In particular, a number of oligonucleotide-mediated site-directed mutagenesis protocols have been developed. A number of different crop/trait combinations are in the pipeline using the patented Rapid Trait Development System (RTDS, registered trademark based on European patent EP 1 223 799, “non-transgenic herbicide resistant plants”). Companies signed co-operational agreements to use the technology in different crops, partially undisclosed, and with different traits, including multitraits.

5.1 Oligonucleotide-directed mutagenesis (ODM)

Oligonucleotide-directed mutagenesis (ODM) is known under various names; for non-exhaustive lists see, e.g., Lusser et al. (2011); Breyer et al. (2009). The technology is a tool for inducing targeted alterations in the genomes of many organisms, including plants, animals and humans.

Oligonucleotides (oligos) target homologous sequences in the genome and induce site-specific nucleotide substitutions, insertions or deletions through repair mechanisms. To achieve this, one or more mismatched base pairs corresponding to the non-complementary nucleotides are introduced into the cells. By this, the DNA sequence may be changed in a targeted manner, or the expression of genes may be regulated. Genomic alterations include the introduction of new genetic information, the reversal of an existing mutation or deletions (BAC 2007). It is also possible to produce “knock out” mutations, e.g. by introducing stop codons, frameshift additions or deletions interrupting the reading frame (Kmiec et al. 2003). In most cases, the target for mutations is one or a few nucleotides. The frequencies of mutagenesis vary vastly, likely depending on the cell type and target locus (BAC 2007).

Oligos such as chimeric oligonucleotides, consisting of DNA and RNA bases (chimeras), and single stranded DNA oligos have been deployed for ODM in plants, primarily for single basepair or frameshift alterations. Most publications report on the successful use of chimeras, and similar approaches have been patented and commercialized. Diverse modifications within the oligos are expected to provide higher resistance against cellular nuclease activity. The chimeras (e.g. chimeric DNA/2’-O-methyl RNA) and modified DNA oligos are self-complementary and designed to pair with a homologous sequence within genomic DNA (Britt and May 2003). Chimeric RNA-DNA oligonucleotides, used to induce the changes intended, contain contiguous RNA and DNA bases in a double-stranded molecule that is folded into a double hairpin conformation (Figure 3). The sequence alteration is effected through the DNA, whereas the RNA gives stability; a homology domain of 5-35 bp is sufficient. Alternatively, single stranded DNA oligonucleotides containing phosphorothioate linkages at the 5’ and/or 3’ end or triplex-forming oligonucleotides can be used. Kmiec et al. (2003) reported that single-stranded DNA oligos containing 2'-O-methyl RNA nucleotides or phosphorothiate linkages were more efficient in conferring specific alterations than unmodified ones or chimeric RNA/DNA molecules. Modifications of the oligonucleotides such as the use of locked nucleic acids (LNA), peptide nucleic acids (PNA), methylation or modifications of the ends of the oligonucleotides increase the binding capacity and prevent rapid degradation. However, to date there is no conclusive decision concerning the optimal design as the efficiencies in all cases are similarly low. Kmiec et al. (2003) concluded that due to interaction of different proteins during the gene alteration event it was not possible to predict the stability of an oligonucleotide based on a given modification.

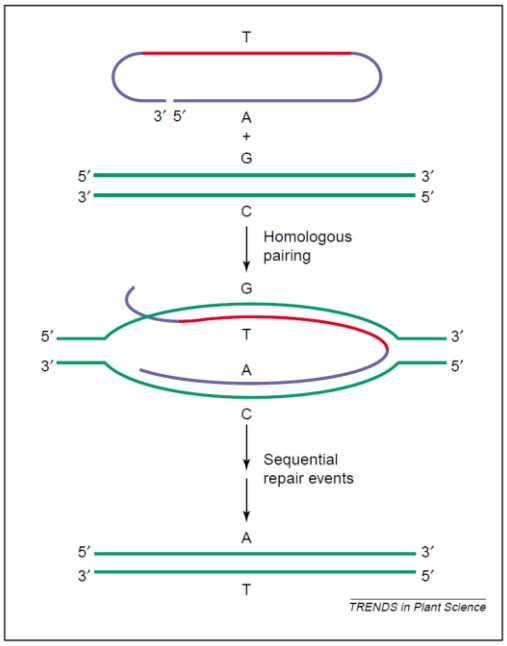


Figure 3. Integration of a targeting oligonucleotide into double-stranded DNA. The chimeric oligonucleotide (RNA: red, DNA: purple) interacts through homologous base paring with the target DNA (green) and results in the formation of a double D-loop structure. The mismatched base pairs are resolved through a series of DNA repair processes. (Britt and May 2003).

The oligos are delivered by methods like electroporation or polyethylen glycol (PEG) mediated transfection. In any case, the oligo is degraded by the cell within hours (ACRE 2011), resulting in transient exposure of cells to the oligo. As the molecules inducing the changes within the genome are degraded by the cell, they are not “heritable”; however, the genomic alterations are heritable.

5.1.1 Limitations in plants

Currently, it seems that neither the efficiency nor the specificity of the technology can be controlled sufficiently. From the data available, several key factors seem to contribute to the successful application of ODM, including the design of the oligo, the sequence targeted, the developmental status of the targeted cell, and the tissue culture and selection system employed. Limiting factors are the stability of the complex as well as the frequency of nonspecific base changes. Both are influenced by the composition of the oligonucleotide (BAC 2007).

Efficiency

High level of efficiency is observed in mammalian systems, whereas in plants the oligonucleotide-mediated gene conversion (mutation) occurs at low frequency (commonly, a frequency of 10^{-4} is given; Britt and May 2003). Mutation rates are thus comparable to those of spontaneous mutations. Spontaneous mutations

obscure the effect of chimeric oligonucleotide-directed gene repair (Ruiter et al. 2003). Zhu et al. (1999) reported that the rate of spontaneous mutations in their negative controls was 10^{-7} to 10^{-8}. On the molecular level, plants obtained through ODM are not distinguishable from those obtained by traditional selection (after mutagenesis or as a result of spontaneous mutation).

The efficiency of ODM depends on the quality of the synthetic oligos, which is a compromise between their length and possible detrimental effects for the cells. Usually, they are in the range of 20 to 30 bp; longer oligonucleotides with lengths up to 100 bp or more have toxic effects on the cells. Also high concentrations of oligonucleotides can be toxic with some types of nucleotide modifications. The small size of the oligonucleotides may contribute to their inaccuracy (BAC 2007).

Unmodified DNA oligos may give low efficiency of gene alteration, likely a consequence of the degradation by nucleases that are present in the reaction mixture or in the target cell. The rapid degradation of the chimeric oligonucleotides within the cell is perceived as a limiting factor for the successful application of the technique.

Apart from the design and quality of the oligos themselves, the different efficiencies reported in the literature may also be due to targeting different cells at different developmental stages (see BAC 2007; Kochevenko and Willmitzer 2003). Both efficiency and specificity may, at least in part, also be due to the sequences targeted. Regularly, semi-targeted, non-specific conversions were observed, and the low level of targeting efficiency precludes addressing non-selectable phenotypes (Zhu et al. 1999; Rice et al. 2000; Britt and May 2003; Kochevenko and Willmitzer 2003). In contrast, some authors claim modification of only the targeted nucleotide (Okuzaki and Toriyama 2004; Rice et al. 2000). It may be hard, if not impossible, to attribute other, unintended sequence deviations unambiguously to the technology. Consistency and reproducibility may potentially be enhanced by further modifications of the effective molecules.

A tissue culture step to regenerate plants from the exposed cells is mandatory. ODM requires the use of protoplast or biolistic transformation, thus apparently the regeneration capacity of the cells is a limiting factor for the application of ODM. The regeneration procedure commonly includes concomitant selection, in particular when the desired trait is herbicide tolerance. Except for herbicide resistance, desirable mutations are difficult to select, even though the possibility to efficiently select targeted plants is a prerequisite to apply the technique successfully. The efficiency to select for mutations, *inter alia*, also depends on the ploidy of the targeted plant. Currently the practical use of the technique is restricted to the selection for herbicide tolerance, in which the cells

can be directly exposed to the selective agent. However, high throughput sequencing techniques allow for the screening of large populations and render the development of plants with other traits possible.

Specificity

ACRE (2011) stated that it is extremely difficult to prove that no other changes have occurred that could unambiguously be attributed to the use of ODM, even by sequencing the whole genome. However, most conventional mutagenesis methods provide no precision and generate numerous indiscriminate changes to the genome.

ODM *via* chimeric RNA/DNA oligonucleotides has been shown to be sequence dependent (Beetham et al. 1999). Beetham et al. (1999) also observed that the modified base in the targeted codon was one nucleotide 5' of the mismatch nucleotide, independent of the oligo used; they attributed this phenomenon to their experimental conditions. Zhu et al. (1999) observed a large proportion of unexpected conversion, which, however, resulted in the desired phenotype. They did not report other mutations within 800 bp of surrounding sequence.

Thus, collateral damage, *i.e.* unintended modifications of other sites, is possible as shown in several reports. Additional research is required to better understand off-target effects, as single mutations can lead to an increase in expressed plant toxins (Kuzma and Kokotovich 2011, and references therein).

5.1.2 Applications and general considerations

ODM may be applied to any given plant if the sequence to be modified, including the effect of the sequence alteration, is known. In contrast to genome modification methods like cisgenesis or transgenesis, no DNA - neither from sexually compatible nor from incompatible species - is inserted; consequently no random integration, multiple insertions or effects due to the insertion in undesirable locations in the genome may occur (Oh and May 2001). The gene remains in its normal chromosomal context, thereby reducing the chances of altered gene expression, unless intended. In common applications, the endogenous expression patterns are expected to persist. Due to presumed target site specificity and sequence dependency of the approach it may be expected that the genomic environment is not markedly perturbed as with transgene integration (Zhu et al. 2000).

Major aims of oligonucleotide directed mutagenesis are, besides the principal target herbicide tolerance, altering fatty acid content (cottonseed, sunflower, rapeseed, linseed, peanut, soybean, maize, rice, etc.), amino acid content, the

production of modified starch, abiotic stress tolerance (drought, increased soil salinity, soil contamination with heavy metals, extreme temperature), male and female sterility; also, the production of mutants for analytical purposes may be an aim (Kmiec et al. 2003).

In particular, genes involved in pathogen defence frequently display rapid evolution through mutation (Niewenhuizen et al. 2012). Consequently, site-directed mutagenesis may be employed to restore activities of, e.g. enzymes (Niewenhuizen et al. 2012), similar to the restoration of an inactive GFP transgene (Beetham et al. 1999).

Generally, the mutation is effected on specific, endogenous genes, and no foreign DNA sequences are involved. ODM is designed to induce precise changes in a gene sequence without incorporating genes from foreign species. The oligos are about 20 to 100 nucleotides long, share homology with the target sequence in the host genome, and are chemically synthesized. Frequently, chimeric oligos are employed (Figure 3). The DNA strand of the chimera likely functions as a template for gene repair – effected by the host DNA repair machinery – while the RNA strand enhances targeting efficiency by stabilising complex formation with the target DNA sequence (Britt and May 2003). Although employing sequences corresponding to the natural plant genomic sequences, they are modified to achieve higher stability in the cell.

As the mutation is inherited regularly, it is easy to manage in breeding programmes (Kochevenko and Willmitzer 2003; Zhu et al. 2000). At least theoretically, it is possible to exchange multiple specific nucleotides until a desired effect is achieved (see Kuzma and Kokotovich 2011). The technique requires shorter development times and is commonly expected to be commercialized more quickly, given that it can be marketed as “non-transgenic” and thereby avoiding regulatory constraints. According to Schaart and Visser (2009), the results achieved with ODM can also be achieved with mutation breeding, such as ionising radiation or chemical mutagenesis, which is exempt from European GMO regulations.

5.2 Zinc finger nucleases for the genetic transformation of plants (ZFN)

Zinc finger nucleases (ZFN) are a relatively new tool for genetic modification. ZFN are a combination of zinc finger proteins that were identified to function as transcription factors in the 1970ies, and appropriate desoxyribonucleases. The most important group of the zinc finger proteins, which are able to bind sequence specific to nucleic acids, harbours one zinc atom in its active centre

coordinated with two cysteine and histidine residues (Cys2His2 type). The nuclease is used to induce a double strand break (DSB) at the binding site.
DSB is a dangerous scenario for cells, and therefore adequate mechanisms have been developed by prokaryotic as well as eukaryotic organisms to repair DNA damage. Two main repair mechanisms exist, non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ repairs DSB by randomly joining the nucleic strands without the use of a template. This mechanism usually comes along with small mutations (insertions, deletions). HR corrects DNA damage using endogenous nucleotides with homologous sequence as a template. By transferring exogenous oligonucleotides into the cells (e.g. using plasmid vectors) that are highly homologous with the targeted DNA, the insertion of vector DNA can be induced. This procedure is therefore suitable for accurate introduction of artificial modifications into the host genome.

Three ZFN methods are usually distinguished (Lusser et al. 2011). Type-1 uses no repair template. The natural repair mechanism (usually NHEJ) leads to point mutations (deletions, insertions of few base pairs). Type-2 uses templates that are homologous to the region of the DSB induced by the ZFN with the exception of specific pair alterations. Therefore, the modification also concerns only a few base pairs, or only one base pair, but in contrast to type-1, is a targeted genetically modification process. Type-3 uses homologous repair templates harbouring gene cassettes which ideally will be integrated into the host genome at the targeted site.

In the context of new plant breeding techniques (Lusser et al. 2011) one can distinguish between transient and permanent effects. Transient effects are due to genetic modifications that do not take place in nucleoid DNA (prokaryotes) or nucleus DNA (eukaryotes) and thus are not stably inherited. Use of ZFN techniques always leads to permanent modifications: in case of type-1 unspecific point mutations, in case of type-2 specific substitutions of few base pairs, and for type-3 the insertion of DNA stretches of several kbp in length.

Targeted insertion of foreign DNA in plants, as well as in animals, is not easily achieved as the native repair mechanism in higher organisms is typically affected by NHEJ and not by HR (Weinthal et al. 2010). This makes site-specific integration of foreign DNA provided as exogenous repair template very unlikely.

However, HR-mediated gene targeting can be enhanced by inducing genomic DSB. In 1996, it was shown for the first time that by using "rare-cutting" restriction enzymes, HR repair mechanisms can be induced in plants and other species (Chiurazzi et al. 1996). ZFNs, for example, belong to this group of "rare-cutting" restriction enzymes. The concomitant use of ZFN and donor DNA homologous to the targeted sequence of the ZFN binding area enables site-specific gene editing in plants with high frequency.

Oligomerized Pool Engineering (OPEN) employs genetic selections in bacteria to identify zinc finger array (ZFA) variants that recognise specific target sequences. ZFAs made by OPEN typically show higher activity than those made by modular assembly, likely because the process of selection accommodates context-dependent interactions among neighbouring zinc fingers in the array.

Of the different types of zinc fingers, type Cys_2His_2 has been found to be best suited for inducing targeted mutagenesis in organisms, since subunits can be designed that specifically recognise and bind sequences of three base pairs. Combining of several subunits increases specificity and enables targeted selection of complex DNA sequences.

In the 1990ies researchers found that the unspecific cleavage domain of the restriction enzyme *Fok*I could be combined with site-specific recognition domains and redirected to certain sites in the genome. As the *Fok*I cleavage domain must dimerise to cut DNA, usually two zinc finger proteins recognising specific DNA sequences left and right of the cutting site are joined with *Fok*I facilitating dimerisation and cleavage (Carroll 2011).

In principle, ZFN systems consist of dimers of zinc finger proteins each of which joined with one *Fok*I domain binding the targeted DNA double-strand. The first standard in zinc finger technology was ZFN systems containing three zinc fingers each. Such ZFN designs can recognise and bind 18 base pairs. More recently, even longer designs have been used, e.g. combinations of dimers of six zinc fingers. Such ZFN designs are able to target and bind 36 base pairs with high specificity (Urnov et al. 2010).

5.2.1 ZFN mutagenesis in commercial crop plants

Genetic modification of crop plants by targeted mutation *via* ZFN techniques is a rather new research area. Although gene targeting experiments with tobacco and *Arabidopsis* plants were conducted in the early 1990ies, it was not before 2005 that studies were carried out on plants using ZFN approaches (Lloyd et al. 2005; Wright et al. 2005).

Generally, the ZFN-1 to -3 techniques are applicable in a wide range of plants including not only main agricultural crops but also vegetables, provided methods for the delivery of the coding genes into plant cells and regeneration of plants from tissue culture are available. The technique is currently mainly used for the breeding of herbicide resistant crops. Further projects could be the application of the ZFN approaches for the removal of antinutrients and allergens through gene knock-out and the removal of antibiotic markers.

More recently, three reports on targeted ZFN mutagenesis of commercial crops like maize, soybean and pea were published. The following section provides a short review of these studies:

1)
Targeted mutation and insertion in maize was described in Shukla et al. (2009). The researchers developed four ZFN pairs to target and induce DSB at the DNA sequences of Ile71 or His100 in exon 2 of the IPK1 locus, which plays an important role in phytate biosynthesis. Therefore, successful disruption of IPK1 via targeted ZFN mutagenesis can be detected by phytate reduction.

The study not only shows targeted disruption of the IPK1 gene, but also targeted insertion of two different gene cassettes carrying the *pat* gene, which confers the herbicide-tolerance trait. Both donor constructs contained short sequences of DNA homologous to the insertion site. The expression cassettes (donor DNA) were directly delivered into embryogenic maize cell cultures via silicon carbide whiskers.

Two different donors were used. One donor carried an autonomous gene cassette, i.e. it contained the rice actin 1 gene promoter (*Act1*). The other donor carried a non-autonomous gene cassette, i.e. it contained no promoter. This strategy was used to distinguish between targeted and random integration of transgene cassettes. It was shown, at the cost of efficiency though, that the use of a non-autonomous gene cassette that harbours no promoter enhances the chances for targeted integration, because expression depends on an endogenous promoter (*IPK1*) and hence on highly specific integration. So, the rates for successful targeted integration of the autonomous and the non-autonomous donor were about 3.4 to 22.1% and 16.7 to 100%, respectively. Additional heritability studies using five transformation events (418-8, 418-6, 418-3, 273 and 419) confirmed the stable inheritance for at least one generation.

2)
An outlook for studying targeted mutation of two pea genes that encode starch debranching enzymes, pullulanase and isoamylase was given in Hussain (2009). Both enzymes hydrolyse different types of starch extracts; isoamylase hydrolyses α-1,6-glucosidic linkages but does not hydrolyse pullulan.

The research group aims at characterisation of the pullulanase and three of the identified isoenzymes of isoamylase for degradation and synthesis of starch in peas. For that purpose ZFN techniques for plant mutagenesis will be implemented. Designed and selected ZFN will be used to induce DSB and stimulate NHEJ in the targeted starch debranching enzymes (pullulanase, isoamylase). This technique should finally lead to the development of knockout

mutants which allow investigation of the exact role of these enzymes in starch metabolism.

Furthermore, Hussain (2009) mention the advantages of ZFN techniques arguing that inappropriate tissue specificity, timing, level and duration of expression can be ruled out, because the targeted gene remains under endogenous control.

3)
In Curtin et al. (2011) targeted mutagenesis in soybean using zinc-finger nucleases was investigated. DICER-LIKE genes (DCL) and other genes involved in RNA silencing (RDR, HEN1) were targeted by eight tandem arrays of three zinc fingers binding highly specific to 18 bp of soybean DNA. The nine targeted genes were *dcl1a*, *dcl1b*, *dcl2a*, *dcl2b*, *dcl4a*, *dcl4b*, *rdr6a*, *rdr6b* and *hen1a*. Three of the ZFNs targeted two paralogous gene copies. It was also shown that hairy-root transformation is a reliable selection method for ZFN mutagenesis.

Initially, the usefulness of the transformation method was tested using a transgenic soybean line harbouring the exogenous GFP gene. In this test it was found that 5 of the 13 amplified samples showed ZFN induced deletions ranging from 27- to 71- bp.

In the next step the nine endogenous soybean genes were targeted. Five of the eight tandem arrays of zinc fingers were found to induce mutations in a total of seven gene targets. The observed insertions or deletions ranged from 1 to 20 bp.

An additional study aimed at investigating the ability to discriminate between the closely related DNA sequences of *rdr6a* and *rdr6b*. The results indicate that highly specifically ZFN mutagenesis was induced at high frequency.

A final test was performed to study the heritability of different mutations induced in two individual plants by ZFN at the DCL4a and the DCL4b locus, respectively. PCR sequence analysis pointed to the fact that both plants were likely heterozygous for their mutated genes. The DCL4a/dcl4a plant showed abnormal phenotype and only two viable seeds could be derived. Heritability with respect to the mutation could not be evidenced. The other mutant (DCL4b/dcl4b plant) produced normal viable seeds, of which 24 seedlings were grown and genotyped. Both, Mendelian segregation pattern (1:2:1) and PCR genotyping confirmed stable inheritance of the mutation, a 2-bp insertion.

One of the 24 plants of the T_1 generation that contained the 2-bp insertion was found to lack the ZFN gene cassette. This could be interesting, since removal of the ZFN construct from the plants genome by normal segregation may play a

role for public acceptance as well as safety reasons (e.g. to prevent additional rounds of mutagenesis).

5.2.2 Limitations

Efficiency and stability

A number of scientific peer-reviewed literature of ZFN transformation studies in plants was screened with respect to frequency rates for successful transformations and genotypic stability of transgenic modifications.

Studies investigating NHEJ-mediated truncated repair (ZFN type 1) in *Arabidopsis* and tobacco plants showed success rates of about 1-10%. From the handful studies currently available, it cannot be deduced which factors influenced the results in what way, but the high variation shows that they were obviously influenced by the different ZFN models applied.

The frequency rates for HR-mediated gene targeting in plants using repair templates (ZFN type 3) were lower. Cai et al. (2009) measured success rates of about 1.7%, but these numbers are based on phenotypic analysis only. De Pater et al. (2009) who studied the frequency rates of ZFN transformation in *Arabidopsis* via PCR analysis found only 3 of 3040 plants (0.1%) showing successful integration of the gene cassette.

These findings indicate that successful transformation of ZFN type 1 (induction of mutations) can be achieved with higher frequencies than transformation of ZFN type 3 (targeted integration of a donor DNA sequence).

As for stable inheritance of the ZFN induced modifications in the plants, all heritability studies were able to demonstrate normal inheritance pattern (Mendelian) or genetic/phenotypic stability of the introduced genomic changes.

a) HR-mediated gene targeting using ZFN technology in tobacco (Cai et al. 2009)

HR-mediated gene targeting in tobacco plants containing a transgenic target sequence with a partial, non-functional *pat* gene was studied using two different ZFN transformation vectors. The donor vectors were integrated in the tobacco genome via *Agrobacterium*-mediated transformation. The two ZFN systems differed with respect to the distance between the ZFN binding site and the homologous sequences. However, exact specification of the used ZFN designs, especially with respect to the number of zinc finger proteins, is not given in the report.

ZFN-1 induced one DSB with ~ 3 kbp non-homologous sequences right and left of the binding site resulting in the substitution of a ~ 6 kbp DNA stretch by HR-mediated DNA repair. The second system, ZFN-2, induced two DSBs ~ 6 kbp distant from each other. This experiment also resulted in excision of a 6 kbp fragment and gene correction via HR-mediated DNA repair. So, successful gene targeting by ZFN for both experimental strategies could be shown by reconstitution of a functional *pat* gene.

The frequency of successful gene targeting in tobacco was phenotypically and genotypically measured. The phenotypic analysis with respect to restored herbicide resistance showed that transformation rates of 1.7% for both strategies were obtained. That is 43 and 47 positive samples out of 214 selection plates in relation to 536 positive control samples.

Genotypic analyses were conducted on PCR fragments containing sequences of both the donor and the target DNA proving the successful, site-specific integration of the donor sequence. All positive samples from phenotypic analysis (43 and 47 samples) were studied, and it was shown that all contained the predicted recombination sequence. An additional nested PCR study on 20 samples revealed that seven of them gave high-fidelity results across the entire integrated sequence.

b) *Agrobacterium*-mediated ZFN transformation in *Arabidopsis* (De Pater et al. 2009)

Gene targeting in *Arabidopsis* plants containing a transgenic target sequence from yeast (chromatin embedded DNA) using *Agrobacterium*-mediated ZFN transformation was studied. Highly specific ZFN design were used consisting of two ZFNs containing six zinc fingers each recognising a total of 36 bp with a spacer sequence of 6 bp. This spacer included an *Eco*RI restriction site.

At first, ZFN transformation with no repair templates was studied. Stable integration of the donor sequence containing the T-DNA (yeast DNA) and both genes encoding for the two ZFNs were investigated by PCR and sequencing. From the three different promoters tested, the Rps5a promoter was found to have much lower expression levels (220-fold) as the two constitutive 35S promoters. *Eco*RI resistance was used to identify T2 generation transgenic *Arabidopsis* plants for successful ZFN transformation events. PCR analysis showed larger deletions (up to about 200 bp) as well as small insertions and deletions (1-14 bp). The frequency of ZFN-induced DSB followed by incorrect repair *via* NHEJ was found to be about 2%.

Secondly, HR-mediated ZFN transformation using a homology carrying construct (repair template) was studied. As selection marker, hygromycin resistance via

integration of the *hpt* gene was chosen. Of the 3040 plants analysed, three were found to contain the expected PCR product, i.e. the complete integration of the hpt coding region by HR. 2860 plants showed no gene targeting events. Further investigations showed that two of the three successfully targeted plants still contained the target locus, the *gfp* gene. Segregation of the hpt locus was evidenced for all three transformants.

c) Transient delivery of ZFN into tobacco and petunia using agroinfiltration (Marton et al. 2010)

For studying non-transgenic production of mutated plants, indirect transient delivery of ZFN into cells of tobacco and petunia using a virus-based (Tobacco rattle virus, TRV) expression system was established. Agroinfiltration was used to inoculate plant cells, and successful infection could be demonstrated by activation of the red fluorescent reporter gene D2Red2. Successful mechanical inoculation was also shown.

Subsequently, co-expression of two reporter genes using two TRV systems was achieved, although with less efficiency than for single expression. In a second step, the expression of two reporter genes using a single pTRV construct was also confirmed. No expression was observed in the control group (non-infected plants).

Transient ZFN expression was then tested using a pTRV vector containing DNA sequences coding for a ZFN system binding the QQR ZFN target site in transgenic tobacco and petunia. Virus ZFN-mediated targeted mutagenesis based on NHEJ-mediated truncated repair using infection *via* agroinfiltration (or by direct delivery of viral virions) could be established in leaves, buds, flowers and even reproductive tissues of plants. No expression was observed in the control group (non-infected plants). Further investigations (PCR analysis) revealed different small insertions and/or deletions at the cutting site. Stability and heredity of the mutations were shown, though quantitative data were not presented. The transient character of the transformation was characterised, since newly developed seedlings (T1 generation) were free of viral particles and thus of vector DNA.

The authors concluded ZFNs to be efficient tools for successful site-specific mutagenesis in plants, but also pointed to the fact that the use of transient DNA-transfer methods may still lead to unwanted traces of foreign DNA in the mutated lines.

d) ZFN-mediated targeted mutagenesis in *Arabidopsis* (Osakabe et al. 2010)

The authors studied ZFN-mediated targeted mutagenesis in *Arabidopsis* using ZFN expression vectors introduced into the plants genome via *Agrobacterium* transformation. The ZFNs were designed to bind the *Abi4* gene as a target gene, which influences plant response to abiotic stress and seed development. Mutagenesis based on NHEJ-mediated truncated repair was investigated.

The used ZFN design consisted of two ZFNs – each fused to *Fok*I cleavage domains - containing three zinc fingers recognising a total of 18 bp with a spacer sequence of 4 bp. The *Arabidopsis* heat shock protein HSP18.2 gene promoter was used to drive the expression of ZFNs. A mismatch-specific endonuclease (surveyor nuclease) was used to detect mutations in somatic cells. The rate for successful mutagenesis in *Arabidopsis* was about 0.26% to 2.86%.

Heritability was studied by randomly selection of seven (from 96) mutated zfn_abi4 lines, and it was found that all seven plants showed a single base deletion at the same position. T3 seeds were also produced to select homozygous mutations. The experiments revealed an expected segregation ratio (1:2:1).

The authors refer to similar findings regarding ZFN-induced mutagenesis, in particular to mutation frequencies of 7% and 5% demonstrated for *Arabidopsis* and tobacco, respectively (Lloyd et al. 2005; Meder et al. 2008).

e) ZFN-induced mutagenesis in *Arabidopsis*, soybean and zebrafish (Sander et al. 2011)

The authors studied ZFN-mediated targeted mutagenesis in *Arabidopsis*, soybean and zebrafish using a publicly available platform of reagents and software for engineering ZFN that is simple to practice (CoDA). The success rate was compared with selection-based methods for ZFN designs such as OPEN.

The mutation frequency induced by the constructed ZFN pairs was 1.1% to 8.4% in *Arabidopsis* plants. Furthermore, CoDA ZFNs induced mutations in a target site present in two duplicated soybean genes in transformed root tissue with rates of 18.8% and 10.7%. The success rates in zebrafish were similar. This success rates are comparable to OPEN ZFN designs.

It is pointed out, though, that one CoDA ZFN in its current form can bind 9 bp target sites, which means in practice that a target sequence of 18 bp at maximum can be specified. Thus, this system cannot produce the same high specificities as, for example, OPEN ZFN, which may still be preferable to those made by CoDA for highly demanding applications. There were no heritability or stability studies performed.

Fidelity of Zn finger nuclease induced DNA repair

The fidelity of Zn finger mediated insertions into the plant genome depends on two factors:

Sequence specificity of protein-DNA binding at the insertion site
Fidelity of the DNA double strand break repair process

ad 1) Zn finger target site specificity

Zinc finger proteins detect typical consensus sequences for binding (Kumar et al. 2006). This implies that these DNA binding proteins do also recognise sequences aberrant from the canonical consensus sequence albeit efficient binding will occur at a significantly lower frequency compared to their primary targets (Paillard et al. 2004; Urnov et al. 2010). The mode of action of zinc finger proteins used in plant gene technology may be compared to the action of transcription factors having weak or strong promoters as reaction partners. Although the zinc finger technology is a substantial improvement concerning target sequence specific insertion of exogenous genetic material this process is still inherently unspecific to a certain degree and may introduce unintended effects due to binding and cleavage at non-target sites in the plant genome. This observation must be taken into account for regulation and risk assessment of organisms produced by this methodology.

Target site sequence specificity may be enhanced by combining up to six zinc finger protein elements targeting 18 base pairs at each side of the restriction site (Urnov et al. 2010).

ad 2) Fidelity of double strand break DNA repair

The synthesis-dependent strand annealing model (SDSA) is usually used to describe the repair of double-strand breaks in plants.

Recombination products predicted by SDSA are depicted in Figure 4. After induction of a double-strand break in the target (Figure 4a), a 3’ single-strand overhang is released by exonuclease digestion (Figure 4b). The 3’ end invades the double-stranded donor to form a D-loop, which can be resolved either through homologous recombination or a combination of homologous recombination and NHEJ (Figure 4c). Products resulting strictly from homologous recombination are generated when the 3’ end of the invading strand is elongated, and homology to the second 3’ end of the double-strand break allows the two single strands to anneal and repair the break (Figure 4 d,e). Non-homologous end-joining comes into play if the 3’ end of the invading strand cannot find complementary sequences at the broken target (Figure 4 f,g).

Lack of complementarity can occur in a variety of ways; for example, exonuclease digestion can remove complementary sequences from the target, or the invading 3' end may copy non-complementary sequences from a donor that lacks complete target homology. This may lead to unintended insertions or deletions at the 3' end of the insert (Wright et al. 2005).

The fidelity of gene targeting was found to be e.g. approximately 20% (Wright et al. 2005).

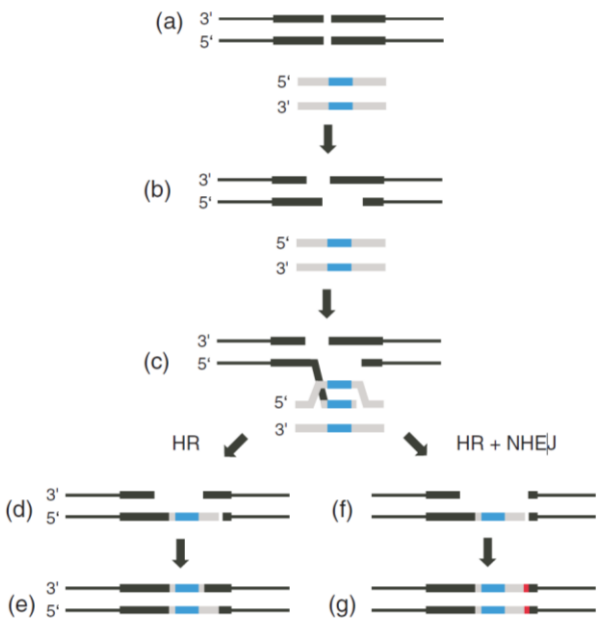


Figure 4. Fidelity of zinc-finger nuclease-assisted recombination (Wright et al. 2005)
(a) A double-strand break is introduced into the target gene by the ZFN.
(b) A 3' single-strand overhang is released by exonuclease digestion.
(c) The 3' end invades the double-stranded donor to form a D-loop. The blue coloured sequences in the donor depict the 600 bp missing from the target locus that are required to restore GUS:NPTII function.
(d, e) Products resulting strictly from homologous recombination are generated when the 3' end of the invading strand is elongated, and homology to the second 3' end of the double-strand break allows the two single strands to anneal and repair the break.
(f, g) If the 3' end of the invading strand cannot find complementary sequences at the broken target, the break is repaired by a combination of homologous recombination and NHEJ. The red sequences denote insertions or deletions that can occur through NHEJ.

Efficacy and potential toxicity

While ZFN-mediated gene modification has been successfully demonstrated in a variety of cells from diverse species like frog oocytes, *Drosophila*, nematodes, zebra fish, mice, rats, plants, and humans, a high rate of endogenous gene modification efficiencies (>10%) have been achieved using this approach, in the case of ZFNs fused to wild-type *Fok*I cleavage domains (FokI_WT), homodimers may also form, which could limit the efficacy and safety of the ZFNs by inducing off-target cleavage. ZFNs toxicity resulting from off-target cleavage, particularly

when using 3-finger ZFNs, has been reported to decrease the viability of targeted cells (Ramalingam et al. 2011).

To test for gene targeting by homologous recombination (HR), Townsend et al. (2009) electroporated plasmids encoding the 815 ZFNs into tobacco protoplasts with donor templates bearing the P191A, W568L or S647T mutations. The mean ZFN-induced herbicide resistance ranged from 5.3% for the P191A donor to 2.4% for the S647T donor.

The surprising outcome of this experiment was that gene targeting frequencies exceeding 2% were obtained at a distance more than 1.3 kb from the cleavage site. This suggests that plant genes can be modified even when DNA sequence composition precludes engineering ZFNs near the desired site of modification.

Furthermore, the authors tested the ability of the 1853 and 2163 ZFNs to stimulate HR and incorporate amino acid sequence changes near their respective target sites. Donor templates were used with mutations in the ZFN target site that prevent cleavage. ZFN 815 was used as a control, and the mutated donor did not substantially alter the overall frequency of herbicide resistance or gene targeting. The mutated P191A donor template did, however, cause an increase in the proportion of gene targeting events at SuRB relative to SuRA. It is unclear why inability to cleave the donor template influences the outcome of recombination. For the 1853 ZFN, the mean number of herbicide resistant events at W568L (281 bp from the cut site) was 0.6% , more than 5-fold lower than gene targeting observed with ZFN 815 at much greater distances from the cut site.

Cai et al. (2009) achieved Zinc finger-mediated gene targeting in tobacco cells, based on the fact that functional herbicide resistance was observed following re-transformation of target events (containing a partial 3' *PAT* gene) with donor DNA (containing the corresponding 5' *PAT* gene sequence) and PCR amplification of expected recombinant fragments was demonstrated. But they observed also imperfect recombination apparently resulting from non-homologous DNA repair.

In a work done by Zhang et al. (2010), toxicity was of particular concern in their targeted mutagenesis strategy, because in contrast to most mutagenesis approaches with ZFNs, they created transgenic *Arabidopsis* plants that have the ZFN expression construct stably integrated. Transgenic plants were also created in the first report of the use of a ZFN in *Arabidopsis*. In the initial *Arabidopsis* study, heat shock-inducible expression systems were used to control ZFN expression. A second report of a ZFN stably integrated into the *Arabidopsis* genome did not describe toxicity when the ZFN was expressed constitutively;

however, in their experiments, they did not recover *Arabidopsis* transformants with certain ZFNs, suggesting that their expression was deleterious.

Transient ZFN expression can potentially be used as an alternative to ZFN-expressing transgenic plants. Indeed, direct plasmid transfer and *Agrobacterium* mediated gene-transfer methods have been the methods of choice for ZFN delivery into tobacco and corn target cells, respectively. Nevertheless, while proven useful for generating ZFN-free mutated plants (as determined by molecular analysis), the use of direct, albeit transient DNA-transfer methods for the delivery of ZFN-expression constructs into target cells may still lead to unwanted and hard to detect traces of foreign DNA in the mutated lines. Thus, even when using transient ZFN expression, crop plants can potentially be classified as transgenic or be subjected to extensive investigation to confirm that they do not possess any traces of foreign DNA within their genome.

The recovery of mutants from transient ZFN expression experiments depends on the ability to regenerate plants from single cells without direct selection, a procedure that has only been successfully applied to a limited number of plant species (e.g. tobacco protoplasts). Therefore, while ZFN technology is a powerful tool for site-specific mutagenesis, its wider implementation for plant improvement may be somewhat limited, by both its restriction to certain plant species and legislative restrictions imposed on transgenic plants. An infection system that can lead to high levels of ZFN expression in a wide variety of plant species, as well as organs and tissues, and that will allow regeneration of mutated and ZFN-free plants is thus needed (Marton et al. 2010).

6 Agroinoculation / Agroinfection /VIGS – Agroinfiltration – Floral dip

Agrobacterium tumefaciens is commonly used for the stable genetic modification of plants. In the case of floral dip or the transformation of somatic tissues a genetic construct is stably integrated into the plant genome. Consequently, plants developed through this technique have to be treated like GMOs falling under Directive 2001/18/EC.

By infiltration of flowering plants ("floral dip method") – in contrast to leaf tissues – the transformation efficiency in *Arabidopsis* can dramatically be improved. Briefly, *Arabidopsis* flower buds (germline tissue) are dipped into an *Agrobacterium* cell suspension to allow uptake of the agrobacteria into female gametes to obtain stable transformation. It requires minimal labour, relatively inexpensive equipment and few specialized reagents, and can be successfully performed. This transformation method is the method of choice to generate transgenic *Arabidopsis* plants in the laboratory. Several protocols for the floral dip method to become less time-consuming and less laborious have been developed for studying gene function in *Arabidopsis thaliana* (Davis 2009). Similar to floral dip, floral spray works well with *Arabidopsis* (Chung et al. 2000).

However, *Agrobacterium tumefaciens* can also be used to achieve transient gene expression in plants. *Agrobacterium* is transferred into plant tissue and stays localised in the infiltrated area. The T-DNA containing construct of the bacteria will be expressed in the plant host but is not stable inserted into the genome of the plant, progeny thereof will remain free of foreign DNA.

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. *Agrobacterium* equipped with this vector is infiltrated into the plant in non-germline tissue like leaves by agroinoculation.

In case of **agroinfiltration/agroinoculation** the expression of the trait/gene is transient and only lasts for a limited period of time. Agrobacteria are transferred into plant tissue and stay localised in the infiltrated area. The T-DNA-containing construct of the bacteria will be expressed in the plant host but is not stably inserted into the genome of the plant; progeny thereof will remain free of foreign DNA.

Agroinfiltration is a technique using *Agrobacterium* as a tool to achieve temporal and local expression of genes in a plant that are foreign to the species. The genes that are introduced in the host cells are usually not incorporated into the plant genome, but rather become temporarily active as free DNA molecules in the plant cell resulting in a rapid transcription into RNA molecules: mRNA in case of genes which are expressed into proteins, or dsRNA when RNAi-constructs are used to block endogenous gene expression (Schaart and Visser 2009).

Transient expression through agroinfiltration (in *sensu stricto*) is a relatively simple procedure. A transgene construct under the control of a tissue-active or constitutive promoter is cloned into a binary vector, which again is introduced into *Agrobacterium*. *Agrobacterium* cell suspensions are infiltrated into leaf panels (non-germline tissue), allowing transformation of accessible plant cells and leading to expression of the transgene(s) contained in the tDNA region. Simple *Agrobacterium*-mediated gene overexpression protocols have now been optimised for several plant species and patents have been filed worldwide (Vaghchhipawala et al. 2011).

The presence and expression of the introduced genes is transient and the gene effect will fade away in time. Expression of the protein starts within a few days after transfer of the gene-construct. From up to 3 – 5 days (Choi et al. 2011) to 5 – 7 days (Baskhar et al. 2009); the Sainsbury Laboratory has a patent (US Patent 7,217,854) on a “High Efficiency Transient Expression System for Plants” where expression persists for up to 12 days post inoculation and longer. It was demonstrated that post-transcriptional gene silencing (PTGS) is the limiting factor in *Agrobacterium*-mediated transient expression in tobacco. By mixing agrobacteria cultures carrying on the one hand a standard binary expression vector for the gene of interest and on the other hand a standard binary expression vector for a suppressor of gene silencing extremely high level expression of the gene of interest can be achieved (Figure 5). Also the expression itself persists for much longer, up to 12 days post inoculation (typically expression disappears after less than five days from inoculation).

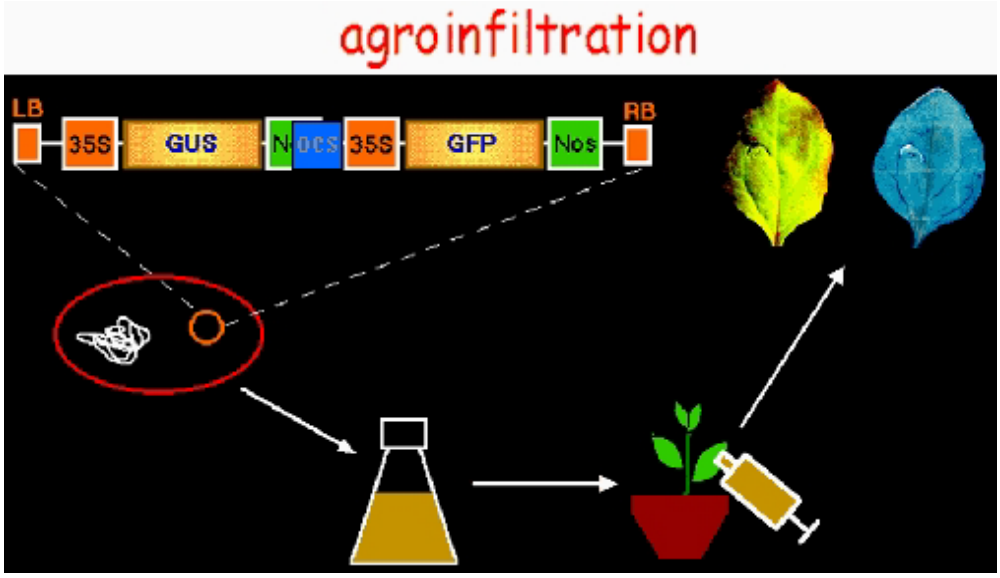


Figure 5. Patent 7,217,854

How long the protein is expressed depends on diverse facts, like

- host plant species
- ages of the host plant: Baskhar et al. (2009) showed that the expression rate in potato leaves at the age of 5-6 weeks is significantly higher compared to leaves which are 3-4 weeks old
- genotype of host plant
- Agrobacterium-strain

Plants differ in their vegetation period; therefore the time of expression is limited by senescence of the infiltrated plant tissue that, in turn, depends on the host plant. Proteins accumulated in the leaves are susceptible to degradation, which is mediated by the endogenous proteolytic activity of hydrolases and interference from phenolic compounds naturally found in these tissues (Vianna et al. 2011).

Agroinoculation (Agroinfection) offers a simple, efficient and powerful approach for delivery of plant viral genomes for understanding viral replication, assembly and movement (Vaghchipawala et al. 2011). VIGS (Virus induced Gene silencing) is a post-transcriptional gene silencing mechanism (PTGS) to transiently suppress endogenous expression of a target gene by infecting plants with a recombinant virus vector carrying a host-derived sequence. Infection and systemic spreading of the virus causes targeted degradation of the gene transcripts (Vaghchipawala et al. 2011). VIGS-vectors are composed of a modified viral genome and include a fragment from the host plant gene to be silenced. Agroinoculation is one of several tools to deliver them into a host plant where inoculation results in replication of the virus and production of dsRNA intermediates. These intermediates are recognised by the plant cell as foreign products, which results in the activation of the plant defence mechanism. The dsRNA intermediates will be degraded into short interfering RNAs (siRNAs),

which lead to specific degradation of mRNAs with identical homology. 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 (Schaart and Visser 2009). VIGS may be used as new breeding technique for silencing certain genes, notably in finding answers to research questions.

Leaf infiltration is the most common method of agroinoculation used for VIGS, however it has limitations (Ryu et al. 2004). A specific and simple method of agroinoculation called agrodrench can be used in even very young seedlings. Here, soil adjacent to the plant root is drenched with an *Agrobacterium* suspension carrying the VIGS vectors, something not possible by the standard leaf infiltration method, which usually requires multiple fully expanded leaves for infiltration. Ryu et al. (2004) showed that agrodrench with a Tobacco rattle virus (TRV)-VIGS-Vector can be used for RNA silencing in diverse Solanaceae species and in young seedlings. Agrodrench is as effective as the leaf infiltration method of agroinoculation for VIGS in *Nicotiana benthaminana* (Ryu et al. 2004).

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. Several studies have shown that *Agrobacterium* is able to move internally through the xylem vessels in grape (Tarbah et al. 1987), and natural pathogenic agrobacteria were able to move systemically inside the plant beyond the site of inoculation for a number of plant species (i.e. tomato, rose, grapevine; Cubero et al. 2006). It cannot be excluded that DNA introduced in the plant tissue can be incorporated into the nuclear DNA, but it is assumed to happen very rarely.

6.1 Applications

Molecular farming

(the production of valuable recombinant proteins in plants and plant cells; Schillberg et al. 2003)

Plant biotechnology relies on two processes for delivery and expression of heterologous genes in plants: stable genetic transformation and transient infection with viral vectors whereas the latter has a number of advantages such as time efficiency, high level of target protein expression, uniformity and consistency of target accumulation, scalability and fewer environmental concerns due to contained facility production (Yusibov and Mamedow 2010).

The most wide spread and important field of application of the methods described above will be found in the field of the production of Plant-made

Pharmaceuticals (PMP). Diverse plants are used as heterologous expression platforms for recombinant proteins, including native and modified therapeutic proteins from humans. In literature a high number of hits describing expression of many different vaccine antigens and plant-made antibodies in many different plant systems have been noted.

The recent biotechnology boom has triggered an interest in utilisation of plants as an alternative expression system for production of vaccine antigens and therapeutic antibodies. As cell cultures of bacterial, yeast, insect and mammalian origin used as expression systems – which have been used for expression of therapeutic proteins – have their limitations, several novel plant expression systems are being explored (Yusibov and Mamedow 2010). The first plant-derived recombinant protein, human serum albumin, was produced in transgenic tobacco in 1990 (Sijmons et al. 1990).

From the pioneering studies aiming at the expression of potentially therapeutic proteins in plants (human serum albumin expressed in tobacco and potato leaves, and suspension cells) emerged the concept of molecular farming, the production of valuable recombinant proteins in plants and plant cells. Plants as expression-host have several advantages over traditional platforms for recombinant protein production. They are inexpensive, highly scalable and do not support human pathogens (Fischer 2012).

Transient expression in order to express alkaloids (codein, morphine, papaverin, noscapine and thebaine) in *Papaver somniferum* has also successfully been done (Hosseini et al. 2011).

A German plant biotechnology company has developed a transfection technology termed “magnifection”. It is a simple and indefinitely scalable protocol for heterologous protein expression in plants, which is devoid of stable genetic transformation of a plant but instead relies on transient amplification of viral vectors delivered to multiple areas of a plant body by *Agrobacterium* (Gleba et al. 2005). Adult tobacco plants, but also many other, in particular edible species, are used for fast production of milligram or gram quantities of recombinant protein for preclinical or clinical evaluation. It has been shown that magnifection works with dozens of proteins tested.

Seed based expression platforms are most competitive in applications that require large volume of recombinant proteins per annum. A second main advantage is that the production of biopharmaceuticals in seeds enables crop production to be decoupled from extraction and purification processes because of the dormancy and storage properties of seeds (Editorial Plant Biotechnology Journal). Several companies are producing biopharmaceuticals in seeds, for instance field-grown rice for the production of human lactoferrin and human

lysozyme (Huang et al. 2008, Zavaleta et al. 2007). A number of plant-derived pharmaceutical products are now very close to the market and because of available processes the first products are entering a clinical trial (Fischer 2012).

Analysis of gene-function

Transient expression of genes using *Agrobacterium* is a powerful tool for the analysis of gene function in plants.
Plant-Pathogen-Interactions: Due to the plants' sophisticated surveillance system they are able to recognise potential pathogens. Specific molecules delivered by a pathogen into the plant cell called effectors are mediated by the products of resistance genes (*R*) present in the host plant. The direct or indirect interaction between a pathogen effector and its matching R-protein triggers a defence signal transduction cascade that results in rapid localised cell death at the site of infection called hypersensitive response (HR). *Agrobacterium* containing candidate avirulence gene(s) (*Avr*) within the T-DNA of the binary vector is agroinfiltrated into the apoplast of a plant harbouring the matching *R* gene. Interaction is based on the timing, occurrence and severity of the HR. Such *Agrobacterium* mediated transient assays have been used in screens to identify resistance genes by co-expression of a candidate R gene which is matching the *Avr* gene in plants (Tay et al. 1999; Bendahmane et al. 2000). Agroinfiltration is used as a tool to improve the understanding of plant disease resistance cascades. Bhaskar et al. (2009) tested *via* agroinfiltration whether a specific gene is associated with potato late blight resistance pathway mediated by the resistance gene *RB*. In this study also the localisation of potato vacuolar invertase (STV-INV) protein to sub-cellular compartments in living cells *via* GFP-expression was monitored. It is shown that this protein was localised in cytoplasm, including endoplasmic reticulum and vacuoles.

Also VIGS used for silencing endogenous plant genes has been successfully used to identify and characterise many plant genes involved in defence against pathogens (Burch-Smith et al. 2004).

Stress-tolerance studies

By delivering the genes of interest into plant leaves and subjecting the leaf segments from the infiltrated area to stress analyses further plant specific traits can be elucidated. A study using tobacco leaves transiently expressing a tomato Phospholipid Hydroperoxide glutathione Peroxidase (*LePHGPx*) gene, showed enhanced salinity- and heat tolerance traits (Chen et al. 2004).

Furthermore analyses in order to determine the role of plant promoters and transcription factors by using agroinfection and inoculation techniques are commonly used. Liu et al. (2011) assessed by *Agrobacterium*-mediated transient expression the inducibility of synthetic promoter constructs *in vivo* in tobacco leaves with the goal to gain further insights into the versatility of an expression system prior to generating stable transgenic plants.

Resistance breeding in plants

Functional analysis of candidate transgenes for insect resistance in stably transformed plants is a time-consuming task that can take months to achieve in even the fastest of plant models. Rapid screening techniques are required. The combination of a robust transient transgene expression assay and a reliable screening protocol could decrease the time required for initial transgene evaluation to determine whether an appropriate phenotype warrants production of stable transgenic plants. The use of transient expression through infiltration of *Agrobacterium tumefaciens* harbouring the transgene and promoter of interest should substantially decrease the time required to test candidate insecticidal genes and might provide a better platform to assess the potential of these gene products. Since there is a wide range of plant species susceptible to *Agrobacterium tumefaciens* infection, the use of agroinfiltration for the evaluation of candidate insect resistance genes has great potential for rapid screening on numerous target insects and host plants (Leckie and Neal Stewart 2011).

The use of this system to evaluate insect resistance genes has several benefits compared to other systems:

The time for preparation of transgenic tissue is drastically reduced – days instead of months (Wroblewski et al. 2005)
The possibility of escape of transgenes into the environment is considerably reduced because the evaluation of results can take place within short time limits (Li et al. 2009)
A system employing agroinfiltration can rapidly be altered for the production of stable transgenic lines (Leckie and Neal Stewart 2011).

7 Cisgenesis combined with other new techniques

In recent years, various new techniques have been developed for the genetic modification of organisms. These techniques complement traditional breeding methods. To date, all techniques are characterised by diverse technical constraints concerning their practical use.

Literature research did not indicate that research and development aim at the combination of cisgenesis with the other new techniques at this point in time. However, potentially all of them could be combined, provided that some preconditions are fulfilled. Most importantly, the techniques envisaged for combination have to be available in the same plant and the combination of traits should be of practical relevance. At the current state of the art, the techniques have been successfully applied only in specific plant species and use only selected traits for which sufficient information is available.

Analysing the currently published information, the combination of new techniques with cisgenesis is not very likely in the coming few years. All techniques have their technical constraints and are used for different plant species. They rely on different methods of transformation and regeneration and, in addition, the currently researched traits have only limited practical utility if combined.

Cisgenesis is defined as the artificial transfer of whole, unchanged genes from same or cross-compatible plant species without inserting foreign nucleic acid sequences neither intentionally nor unintentionally. Attempts to develop cisgenic plants largely focus on fruit trees (in particular apple) and potatoes. In these plant species, the major traits of interest are disease resistances (potato late blight; fire blight, scab resistance in apples) and, less frequently, quality traits (anthocyanin accumulation to achieve attractive red apples). Cisgenesis is most suitable to transfer single genes. Many important traits in plants are governed by more than one gene, which has to be taken into consideration when applying the concept for variety improvement. Genetic interactions have to be identified in order to use the concept with several genes, and the potentially changed behaviour of genes in a different genomic context has to be taken into consideration.

Oligo-directed mutagenesis (ODM) commonly leads to (point) mutations of endogenous genes without inserting foreign sequences. Site-directed mutagenesis opens up vast possibilities of modification if candidate

genes/proteins are characterised. The sequences of targeted genes and functional characteristics, together with the effects of a specific mutation, have to be established. Further restrictions applying to site-directed mutagenesis techniques like for ODM or ZFN-1 and -2 are that 1) the function of a specific gene has to be known, and 2) which sites within the sequence are responsible for a specific function. One of the most important prerequisites for the use of ODM is that suitable regeneration protocols are available for the plant species at hand (Lusser et al. 2011). ODM has as yet primarily been used to induce herbicide tolerances, mainly in rapeseed/canola, maize, rice, potato, sorghum, soybean, and wheat. Further approaches include quality traits like fatty acid or amino acid contents, abiotic stress tolerance, or the restoration of enzyme functions. Like for cisgenesis, a major breeding goal is resistance against *Phytophtora* ssp.

Combination of cisgenesis with ODM

Similar to endogenous genes previously inserted, cisgenes can be targeted and mutated, thereby changing their properties and/or expression. It would be less attractive to transfer mutated cisgenes by traditional cross breeding. It is reasonable to speculate that *Phytophtora* resistance in potato may be engineered using both cisgenic and ODM-based approaches. Using a multiple approach targeting several genes may facilitate that newly created resistances are not broken rapidly.

In all plants, for which an efficient protoplast regeneration protocol is available, the induction of herbicide tolerance through ODM is feasible.

ODM-induced alterations of a cisgene, including its regulatory elements may lead to enhanced expression of the modified gene. If applicable and determined based on an appropriate analysis this should be considered during the risk assessment. Depending on the trait the environment but also food and feed safety may be affected.

The cisgenic modification is easy to detect due to its unique insertion site, given that information on the insertion site is available. If, in addition, the cisgene or another endogenous gene is modified through ODM, the same restriction in detection and quantification of the ODM-induced mutation as described in the corresponding sections of this report apply. Briefly, information on the mutation has to be provided to render detection possible whereas it may be difficult to develop a suitable quantification method.

Zinc finger nucleases (ZFN-1 and -2) are used to create small mutations, whereas ZFN-3 allows for the insertion of larger stretches of DNA. The successful use of zinc-finger nuclease techniques is currently restricted as plants

have to be regenerated from single cells, and corresponding protocols are not available for all plant species. In addition, an efficient selection system has to be available in order to select for those plants that carry the desired mutation and show the intended phenotype. Therefore, gene-specific mutagenesis to date is almost exclusively restricted to selectable phenotypes, like tolerances and resistances against herbicides, abiotic stress, or pests. As the different techniques may be used for different types of modifications, the goals may be manifold. Like for ODM, the function of a specific gene and the effect(s) to be expected after mutating particular sites have to be identified.

Combination of cisgenesis with ZFN

Potential combinations of ZFN-1 and ZFN-2 with cisgenesis are similar to those combining ODM and cisgenesis. A promising new tool in genetic engineering of plants that can potentially be combined with cisgenesis on a large scale is ZFN-3, which stimulates the integration of DNA stretches of several kbp in length using homologous repair templates. Thus, the targeted integration of "cisgenes" making use of a ZFN cleavage mechanism could be a meaningful instrument for genetic modification of plants. The targeted integration of the gene of interest into the genome would minimise risks associated with currently available transformation methods, in particular the integration of a gene into another genomic context. A cisgene could be inserted precisely into the desired site while the linkage drag usually observed with traditional cross breeding methods is avoided. Moreover, with the ZFN type-3 technique and thus targeted DNA insertion the issues raised when applying *Agrobacterium*-mediated transformation are no more relevant.

The state-of-the-art shows that the ZFN techniques potentially cause detrimental off-target mutations. Due to insufficient specificity and efficiency the application of the ZFN techniques in plants is not expected to be successfully (*i.e.* without any unintended effects) combined with cisgenesis in the next years. As ZFN is intensively researched, rapid progress may be expected and future developments will have to be observed, in order to carefully assess opportunities and risks associated with this new technique.

Agroinfiltration

In **agroinfiltration/agroinoculation** agrobacteria are locally infiltrated into plant tissue and lead to the transient expression of a trait/gene without stable insertion into the genome. Among the diverse applications, the production of plant-derived pharmaceutical products is the commercially most attractive. Processes for the production are documented and products have entered clinical trials (Fischer 2012). Agroinfiltration is also used for the analysis of gene function, or stress tolerance studies.

Combination of cisgenesis with Agroinfiltration

As agroinfiltration is applied for very specific purposes (molecular farming, analysis of gene function and stress tolerance) its combination with cisgenesis may be anticipated in single cases only. However, the evaluation of candidate genes potentially used for cisgenic approaches could be clearly accelerated by agroinfiltration, as it allows quick evaluation of candidate genes potentially useful for cisgenic approaches. Nevertheless, to date literature research does not give any indication about intended combinations of the techniques.

8 Traditional plant breeding techniques

Plant breeding aims at the improvement of agronomic, quality or processing characteristics or higher levels of resistance against environmental or disease impacts, commonly referred to as value of cultivation and use (VCU) of plant varieties. The breeding goals are approached by different methods.

Cisgenic plants are frequently claimed to be similar to traditionally bred ones (e.g. Schouten et al. 2006). However, the consequences on the genomic level resulting from the application of traditional and new plant breeding techniques depend on the method applied. Whereas when producing cisgenic plants the genomic context within the plant is altered, it is likely that plants resulting from targeted mutagenesis (via oligonucleotides, zinc-finger nucleases, or meganucleases) are similar to plants after conventional mutation breeding. Moreover, most of the genomes mutagenized in a targeted way are expected to display fewer alterations than observed after conventional mutation breeding.

The development and application of the modern techniques to create genotypes with targeted characteristics is at least in part due to the limitations with traditional plant breeding. With traditional methods, the use of genetic resources in plant breeding programmes is time-consuming and laborious. New technologies, like cisgenesis and intragenesis, but also oligonucleotides, zinc-finger nucleases, or meganucleases, may overcome limitations with traditional plant breeding methods, such as long breeding time, linkage drag, crossing barriers, and may be an efficient way or even the only alternative to obtain a desired genotype. The breeding for polygenic traits, however, is still hampered by the need to have the genes that are involved in the manifestation of the traits characterised, isolated, and readily available for transformation. The transformation itself may not be easily achieved as several genes need to be stacked, either directly in the construct or by crossing plants harbouring the desired traits.

In traditional plant breeding, the new genotypes are either found by selection breeding, by cross breeding or by mutation breeding programmes. Plant genomic research broadens the tools available to researchers and traditional plant breeders, as more and more complete plant genome sequences are published (Pennisi 2011). This steadily advancing knowledge facilitates the efficient identification of candidate genes involved in the manifestation of the desired traits. Also, the number of techniques available to traditional plant breeders has impressively increased in recent years. These techniques include linkage mapping, association genetics/mapping, nested association mapping

and high-throughput marker genotyping (screening) platforms. Although many breeders and R&D departments of companies exploit the increasing knowledge on plant genomes, the new approaches may only assist classical plant breeding methods, which are still widely employed and will also be an indispensable part of future breeding programmes.

Marker-assisted techniques have greatly contributed to the faster selection of desirable individuals in breeding selection schemes (Collard and Mackill 2008). Marker-assisted recurrent selection (MARS) or marker-assisted backcrossing (MABC) may speed up the identification of the desired individuals after hybridization to introgress candidate QTLs or genes in elite lines. Molecular breeding increases the selection efficiency and thereby enhances genetic gain (Varshney et al. 2011). Marker-assisted breeding, particularly high-density marker collections, greatly increased the predictability of breeding efforts, in which crossovers are and will remain crucial (Winjnker and de Jong 2008).

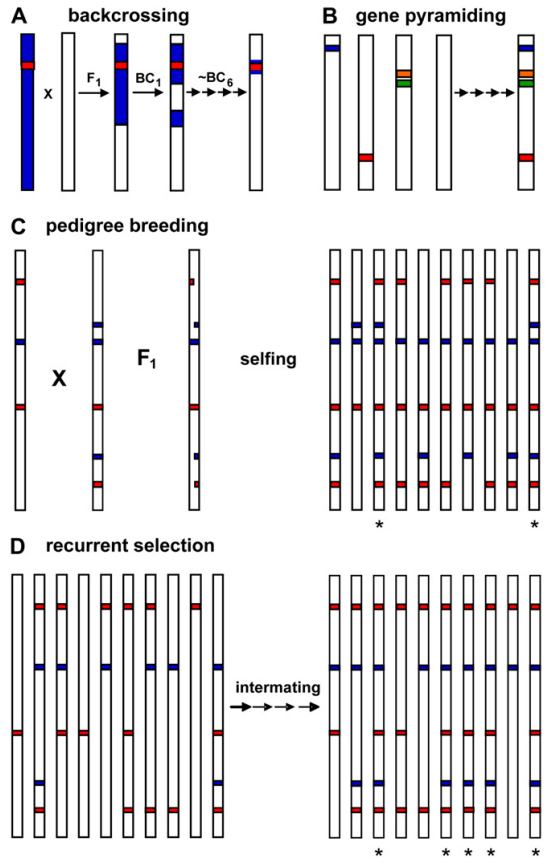


Figure 6. Common breeding and selection schemes; coloured segments indicate genes and/or QTLs within a genome (vertical bar) that influence traits under selection. A, Backcrossing. A donor line (blue bar) featuring a specific gene of interest (red) is crossed to an elite line targeted for improvement (white bar), with progeny repeatedly backcrossed to the elite line. Each backcross cycle involves selection for the gene of interest and recovery of increased proportion of elite line genome. B, Gene pyramiding. Genes/QTLs associated with different beneficial traits (blue, red, orange, green) are combined into the same genotype via crossing and selection. C, Pedigree breeding. Two individuals with desirable and complementary phenotypes are crossed; F1 progeny are self-pollinated to fix new, improved genotype combinations. D, Recurrent selection. A population of individuals (10 in this example) segregate for two traits (red, blue), each of which is influenced by two major favourable QTLs (Moose and Mumm 2008).

The hybridisation (cross pollination) with specific trait carriers (e.g. breeding lines, varieties, genebank material, wild types) may lead to progenies containing the targeted genotypes. Commonly observed limitations in cross breeding are linkage drag and crossing barriers. The selection and homocygoting process that starts in the segregating F2-generation (according to Mendelian rules) for traits that can be evaluated on a single plant basis is based on different methods, like the pedigree or bulk method, and will take several years. A conventionally bred variety is produced in about 8 to 10 years. In trees, the breeding procedure may take several decades.

By definition, **linkage drag** is “the reduction in fitness in a cultivar because of the introduction of deleterious genes along with a beneficial gene during backcrossing.” (Feuillet et al. 2007) In donor plants carrying a gene coding for a desired characteristic – e.g. tolerant crops, landraces or wild relatives (Varshney et al. 2011) – the gene of interest may be linked with genes responsible for severe losses in performance or quality. This phenomenon is frequently observed when using wild relatives, as they carry a lot of traits not adequately

adapted. Linkage drag may be erased by backcrossing with the highly developed parent and repeated selection for the desired trait. Backcrossing is time-consuming and expensive, as usually five backcrossing steps are needed to get the portion of the foreign genes in the genome below 2%. However, in vegetatively propagated trees and vines, in particular when employing highly heterozygous varieties and long generation times, backcrossing to transfer an engineered trait cannot be employed.

When a cross is made between a recipient crop and a related species carrying a trait of interest, the linkage drag usually occurs between homologous segments, *i.e.* chromosomes from the two different species. Homoeologous chromosomes have a higher degree of sequence divergence than homologous chromosomes. The following recurrent backcrosses narrow down the introgressed segment due to crossovers between the recipient and the introgressed homologous region. Through this natural mechanism during regular meiotic chromosome pairing the gene of interest is retained and "wild" undesired genes resulting from the linkage drag are removed (Winjnker and de Jong 2008). During meiosis chromosomes and chromosome segments recombine, giving rise to an infinite number of genotypically different offsprings. The success of backcross breeding, *i.e.* the reconstitution of the elite genotype with its positive traits including the new, introgressed trait, depends on the structure of the locus of interest in which the desired gene is located. As the occurrence of crossovers is in part determined by higher order chromosome structure, some regions are not subjected to crossover recombination. Consequently, loci in such regions remain linked to different extents (Winjnker and de Jong 2008).

A major difference as compared to gene insertions using current standard plant transformation methods is thus the introgression of the gene within a similar genomic context.

Natural **crossing barriers** may prevent that the carrier plant for the desired gene or genes is crossed with the high bred variety. Special techniques are then necessary to force plants to interbreed such as cell culture techniques (e.g. embryo rescue). The transformation methods used to produce cisgenic or intragenic plants allow for the introduction of genes directly into an elite genotype. However, the methods are the same as for the production of transgenic plants.

The performance of plants in important traits – or what is perceived as one trait by the breeder, producer or consumer – is often the result of a complex physiological and genetic system. **Polygenic traits** such as polygenic disease resistance, winter hardiness, drought resistance, complex quality traits or yield potential itself are influenced by many single genes and their interaction. Also genotype-environment interactions may play a significant role. Specific

knowledge about the genetic background of potential crossing partners is necessary. Where this pre-information is not available, breeding work on such complex traits should be based on large populations and accurate observation methods to be successful in the selection of the adequate genotypes. The breeding for polygenically inherited traits may not be facilitated by applying cisgenic methods. The genes responsible for the traits must be known and available, the interaction of the genes within their natural context must be taken into account, and also the number of genes that may be introduced into a cisgenic plant is limited due to technical constraints. The combination of traits may in many cases again only be achieved through traditional crossing methods.

Besides traditional selection or crossing programmes, conventional plant breeders have been using many other techniques to develop new varieties (Akhond and Machray 2009). When applying **mutation breeding** techniques, huge populations are required to increase the probability that the desired genotype will arise. Efficient screening and selection methods are indispensable to identify the genotypes that fit the breeding goals. The prime strategy in mutation-based plant breeding has been to create variation that could not be found before in a population, and to improve important traits in existing varieties (*i.e.* to upgrade well-adapted ones) by altering one or two major traits, in particular yield and quality traits (Ahloowalia and Maluszynski 2001). The mutation may be spontaneous, induced, or derived from recombination-based breeding, and the mutants are either directly released as new varieties, or used as parents, but also as tools in genome research. In Europe, mutation breeding primarily has aimed at improving crops for the processing industry, e.g. sunflower, rapeseed, linseed, or barley.

In the past, numerous traditionally bred varieties have been developed from induced mutations with radiation and chemicals. In addition, many traits are due to natural mutations (Wilde et al. 2012). Mutated genes have been freely used in the development of many varieties with desired traits all over the world, and in Canada, Australia, and also Europe, many cultivars are based on mutant germplasm (Ahloowalia et al. 2004). More than 2,000 mutant varieties have been officially released, in particular in Asia, and less frequently in Europe and the Americas (Ahloowalia et al. 2004). The FAO/IAEA Mutant Variety Database (MVD) provides technical details of crop mutant varieties including the mutagen used and the characters improved (http://nucleus.iaea.org/CIR/CIR/MVD.html#). As naturally (spontaneously) arising mutations occur rarely, mutations in plants have been induced employing gamma rays, fast neutrons, or mutagenic chemicals like ethylmethane sulphonate (EMS), menthylmethane sulphonate (MMS), hydrogen fluoride (HF), sodium azide, N-methyl-N-nitrosourea (MNU), and hydroxylamine.

Herbicide tolerance may be achieved without inserting foreign DNA but derived from selection or mutagenesis. The spontaneous appearance of herbicide-resistant weeds (*via* mutation) is a recognized phenomenon for all classes of herbicides, in particular, when the herbicide is applied at low rates (Beckert et al. 2011). Tolerance to herbicides, in particular sulfonylureas and imidazolinones, is based on mutations in acetohydroxyacid synthase (*AHAS*) genes. In Europe, currently cultivated herbicide tolerant varieties (e.g. oilseed rape and maize) have been obtained by selection of spontaneous variability or by mutagenesis. Oilseed rape varieties resulted from the mutagenesis of microspores; wheat and rice were developed from chemical mutagenesis of seeds, whereas imidazolinone-tolerant sunflower was obtained by selecting naturally occurring tolerant mutants in wild sunflower and transferring the trait to cultivated types (Tan et al. 2005). Mutagenesis is currently excluded from the scope of the EU GMO regulatory framework:

Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:
(1) mutagenesis (according to Annex I B, Directive 2001/18/EC)

Therefore, to date, cultivars developed through mutation breeding have not been subject to GMO-related regulatory constraints. Consequently, cultivars developed through targeted mutation breeding are currently subject to legal regulations valid for conventionally bred cultivars (Wilde et al. 2012). Seed from such plants is not classified as GM, but defined as being a result of “classical plant breeding”. Conventionally bred and currently commercialized imidazolinone-or sulfonylurea-tolerant crops (e.g. Clearfield, Express Sun) are non-transgenic and marketed as non-GMO (Beckert et al. 2011).

9 Plant transformation

(If not otherwise stated the information is retrieved from Conner et al. 2007)

Transgenic and cisgenic plants are produced by the same transformation techniques (Schouten et al. 2006). For the production of most of the cisgenic plants *Agrobacterium*-mediated transformation currently appears to be the method of choice for cisgenic modification. The use of biolistic transformation is documented less frequently (Akhond and Machray 2009, Lusser et al. 2011).

9.1 Biolistic transformation

Vector DNA containing the gene of interest and a marker gene are adsorbed onto inert metal microprojectiles (gold/tungsten) and are shot onto plant tissue via particle gun acceleration. The technology was initially developed by Sanford and co-workers applying gunpowder charges (Sanford et al. 1993). Nowadays the biolistic system is mostly powered by a burst of helium gas (Akhond and Machray 2009).

The most intriguing advantages of microprojectile bombardment are the highly successful application of the particle gun method in transforming monocotyledonous plants, the fact that biolistic transformation is simple, efficient and of similar methodology for all targets, in particular its high versatility and adaptability to a wide range of cells and tissues, and that biolistic transformation is genotype-independent (Southgate et al. 1995, Akhond and Machray 2009).

Major disadvantages of biolistic transformation are its low transformation rates, frequent vector backbone integration into the plant genome, transgene cassette disintegration, multiple copy inserts and gene silencing. In addition, special equipment is needed and the method is cost-intensive (Akhond and Machray 2009, Barampuram and Zhang 2011).

9.2 Transformation using *Agrobacterium tumefaciens*

Agrobacterium tumefaciens, hereafter referred to as *Agrobacterium*, is a pathogenic soil bacterium inducing crown gall or hairy root development (Gelvin 2008, Conner et al. 2007). It is an extremely useful tool due to its capacity of trans-kingdom DNA transfer (Tzfira et al. 2004). Successful DNA transfer to a broad range of different recipients is documented, e.g. prokaryotes (Kelly and Kado 2002), yeasts (Piers et al. 1996), fungi (de Groot et al. 1998, Goukda et al. 1999), and mammalian cells (Kunik et al. 2001).

The transformation process is mainly based on tissue-culture techniques, and no special equipment is needed. For the generation of transgenic plants, *Agrobacterium* was initially used only for dicotyledonous species. Nowadays, also successful protocols applicable for monocotyledonous plants are available (Sood et al. 2010).

Agrobacterium-mediated transformation is currently the method of choice for the genetic modification of plant cells. The development was supported and accompanied by continued improvements of plant tissue culture and a better molecular understanding of the underlying T-DNA transfer processes (Barampuram and Zhang 2011). The efficiency of transformation ranges from low to high according to plant species and cultivar (Lusser et al. 2011).

9.2.1 Molecular characteristics of the transfer process

The central genetic element is represented by an episomally encoded T-DNA ("Ti-plasmid"). This T-DNA encodes genes for transfer (*vir*), tumour formation, and opine biosynthesis functions (Conner et al. 2007). The T-DNA sequence on the Ti plasmid is delimited by short direct repeats of approx. 25-bp (left and right border, LB, RB).

DNA transfer is initiated by induction of the *vir* region of the Ti plasmid and *Agrobacterium* chromosomal genes by plant specific signals (monosaccharides, phenolic compounds). Two Vir proteins (VirD1/VirD2) act as site-specific endonucleases, cutting the bottom strand of the T-DNA borders and releasing a single stranded T-DNA. This T-DNA strand – covered by ssDNA binding proteins – is transported into the plant host cell via a channel. In the host cytoplasm, the T-DNA strand is covered by additional host proteins and the resulting nucleoprotein complex is transported into the nucleus. Insertion is mediated by various plant specific factors, involvement of bacterial Vir proteins and takes place merely at random positions. However, microhomology between T-DNA border sequences and plant pre-insertion sites have been recognised. Polarity of insertion is mediated by the VirD protein attached to the 5' end of the single stranded T-DNA right border. (Tzfira et al. 2004)

A major step forward was the development of disarmed *Agrobacterium* strains where the genes responsible for tumour formation or the whole T-DNA were eliminated. Cointegrate and binary vector systems allowed the separation of T-DNA from *vir* genes onto two different plasmids.

Major advantages of *Agrobacterium*-mediated transformation are that large intact DNA molecules can be transferred. The method allows a high frequency of single locus insertions and the copy number of the inserted gene construct(s) is usually low. Stable integration is more often achieved as compared to other

transformation methods, and a stable expression over generations is more easily achieved (Conner et al. 2007, Barampuram and Zhang 2011).

9.2.2 Disadvantages of *Agrobacterium*-mediated transformation

(compiled from the following sources: De Buck et al. 2000, Wood et al. 2001, Lange et al. 2005, Filipecki and Malepszy 2006, Wilson et al. 2006, Kok et al. 2008, Ulker et al. 2008, Gelvin 2009, Petti et al. 2009 and references therein, Oltmanns et al. 2010, Barampuram and Zhang 2011)

Some of the drawbacks of *Agrobacterium*-mediated transformation are that – using a T-DNA based transformation technology – cisgenic plants contain short non-coding bacterial border sequences. At least 3-4 nucleotides from the right T-DNA border sequence are transferred into the plant genome. Due to the imprecise nature of T-DNA integration (nicking of the left border T-DNA) at the left border site frequently non-T-DNA sequences from the vector backbone are integrated into the plant genome. Such integration of vector backbone sequences may lead to microhomology between a transgenic plant and a microorganism. Thus, Ti plasmid sequences outside the borders may occasionally be transferred. Moreover, *Agrobacterium* C58 contains a linear and a circular genome, and parts of the bacterial chromosomal DNA has been observed to be transferred with the plasmid derived T-DNA. This leads at some instances to a series of intact bacterial open reading frames inserted into the plant genome. The same likely applies to other strains commonly used for the transformation of plants (e.g. EHA101/105, AGL-0/-1), as they are derived from *Agrobacterium* C58. T-DNA integration into the plant host genome cannot be targeted but preferentially occurs in highly transcribed regions which may give rise to unintended insertional mutagenesis.

The integration of vector backbone sequences during *Agrobacterium*-mediated transformation has been shown to occur frequently in virtually all plant species amenable to this transformation technique. In addition, this phenomenon has been demonstrated to be genotype dependent using commonly used *Agrobacterium* strains.

When generating transgenic plants, the aim is to identify insertion events that have a single intact T-DNA inserted into a DNA region with no currently known function. The event should not display any deletions, rearrangements, and no insertion of superfluous DNA.

The majority of primary transformants produced by *Agrobacterium*-mediated transformation show multiple copies of the T-DNA inserted at one or more loci or truncated T-DNAs.

The insertion sites of recombinant DNA, including cisgenes, currently cannot be targeted and thus the insertion occurs randomly in the genome. This may alter and even inactivate the expression of endogenous genes. In addition, the insertion site may influence expression levels or patterns as well as the stability of the transgene (insert). If the recombinant DNA is integrated into an unstable locus the transgene will typically exhibit non-Mendelian inheritance. Insert stability may also display species dependency. In addition, the dynamic nature of plant genomes makes them prone to unintended effects unrelated to the target traits.

Preference for T-DNA integration in highly transcribed regions has been shown. The left border T-DNA sequence may be nicked imprecisely, which leads to the delivery of non-T-DNA sequences from the vector backbone. Nicking of the left border T-DNA sequence can occur in an imprecise fashion leading to the delivery of non-T-DNA sequences from the vector backbone.

Also the epigenetic (genome-wide) variation in plants, which may besides many other factors also be caused by the insertion of additional sequences or the necessity to culture the plant cell *in vitro*, is a well-studied phenomenon. It may lead to unintended changes within the genome of the transformants. Its manifestation *inter alia* also depends on the actual sequence of the inserted gene and the insertion site. Interactions with different genetic backgrounds are possible and may result in unexpected consequences.

Most transformation methods today rely on more or less extensive regeneration procedures, frequently also involving selective agents and plant growth regulators. Prolonged *in vitro* phases may lead to unintended changes in the plant genome, a phenomenon well documented as somaclonal variation. The primary transformants resulting from the use of tissue culture steps will thus frequently not only harbour the intended modification but also other unknown variations. These are not due to the transformation process itself but result from the tissue cultures techniques. The methods of regeneration vary depending on the explant and plant species, and may also be genotype-dependent. Hence it is difficult to predict which changes occur within a given plant genome.

To overcome transformation- and/or regeneration-induced mutations, successful plant transformation relies on rigorous selection and subsequent breeding programmes, similar to traditional strategies.

Sequences called “filler DNA” may comprise up to several hundreds of base pairs. Such sequences originating from plant DNA close to the insertion site and/or sequences from near the T-DNA ends close are found frequently in

transformants. Besides the insertion of additional sequences, also deletions in the plant genome have been observed.

Without complete sequences of new events and the host plant it is not possible to determine whether there are additional small insertions of T-DNA, plasmid sequences, deletions or rearrangements of genomic DNA. The stability of transprotein expression should be verified throughout several generations. Southern blot analysis has to be endorsed by sequencing to identify mutations created during the transformation process.

Sequence information will also aid the identification of the exact genetic construct that was inserted into the host plant genome and to assess the possibility of insertional mutagenic effects.

Optimised transformation techniques to minimise the time in culture as well as successful adoption of targeted insertion techniques (e.g. through homologous recombination) are expected to render plant transformation safer and more specific in the future.

The integration of vector backbone sequences during *Agrobacterium*-mediated transformation has been shown to occur frequently in virtually all plant species amenable to this transformation technique. The backbone integration also depends on the genotype in commonly used *Agrobacterium* strains.

As all currently published articles reporting cisgenic approaches used *Agrobacterium* for the transformation process, the same risks apply to cisgenic plants, similar to intragenic, plant-based vectors.

9.3 Plant transformation vectors

9.3.1 Conventional vectors for the generation of transgenic plants

The essential components of conventional transformation vectors (T-DNA, recombination sites, selectable marker genes, and a bacterial multiple cloning site for insertion of the gene of interest) are usually of bacterial origin.

Transfer of superfluous vector backbone DNA into plant genomes is to be avoided according to current EFSA guidelines (EFSA 2006; EFSA 2011). However, in many cases vector backbone sequences are transferred. This may be avoided to some extent by using novel gene delivery and vector systems (Oltmanns et al. 2010; Ye et al. 2011) or by elimination from the ongoing breeding programme. Selection against these transformation events containing vector backbone DNA are facilitated by PCR screening for backbone sequences and simultaneous targeted selection strategies, e.g. by insertion of the *barnase* suicide (Hanson et

al. 1999) or *CodA* genes in the vector backbone (Stougaard 1993) or the insertion of reporter gene constructs in the backbone: ß-glucuronidase, isopentenyl transferase. These targeted strategies do not necessarily identify all backbone sequences.

By using minimal T-DNA vectors the amount of superfluous DNA can be reduced to a minimum (Barrell and Conner 2006). They contain a very short T-DNA stretch with a tightly inserted selection marker and several restriction sites for inserting the gene of interest between left and right border sequences. Moreover, efficient integration is possible using only a single border in the right border orientation. Deletion of the left border has only minimal effects on the efficiency of T-DNA transfer.

9.3.2 Intragenic vectors

The concept of intragenic vectors is a major extension of the minimal T-DNA vector system (Rommens et al. 2010). Intragenic vectors consist only of plant-derived DNA from crossable species and are therefore considered for the generation of “non-transgenic GM crops”.

The minimal requirements for an intragenic vector are that the plant-derived T-DNA-like region contains at least one border sequence (RB, right border) and a multiple cloning site for insertion of the gene of interest, an origin of replication (for maintaining the vector in *Escherichia coli* (*E. coli*) and *Agrobacterium*) and a selectable marker (for maintaining the vector in *E. coli* and *Agrobacterium*) (Conner et al. 2007).

The ultimate goal is to develop vectors containing only plant derived DNA sequences (=P-DNA). Vectors are available which use DNA sequences from the same or related crop species to insert target genes (Rommens et al. 2005).

P-DNA (Conner et al. 2007)

P-DNA elements should provide sufficient homology to T-DNA sequences to allow insertion into the plant genome, but have to be of plant origin. Bacterial or viral DNA is to be absent.

A complete T-DNA similar fragment was successfully achieved in potato by PCR analysis of pooled DNA sequences from 66 different varieties of potato. Degenerated T-DNA border specific primers allowed the isolation of a 391-bp amplicon flanked by sequences showing sufficient similarity to bacterial T-DNA border sequences. However, the chance for detecting such elements in plants is extremely low (Barrell et al. 2010).

The chance for the detection of a single T-DNA border sequence in a plant genome is substantially higher. The search for homologous plant genome sequences corresponding to a T-DNA border core motif of 12 nucleotides was extremely successful. Appropriate sequences were detected in tomato, potato, petunia, tobacco, apple, rice, onion and pine.

Given the fact that only three to four nucleotides of the bacterial right T-DNA border are integrated into the plant genome, it is feasible to construct a vector containing these three to four nucleotides of plant origin. The remainder of the vector may be of prokaryotic origin. Proof of principle for such a kind of chimeric right T-DNA border sequences was achieved by using *Arabidopis thaliana* genomic DNA which contains a single T-DNA like border sequence. Via specific PCR primers a 23-bp fragment of a bacterial binary vector was annealed to the appropriate four plant nucleotides. This construct was successfully ligated into the backbone of a bacterial binary vector, which was used to transform *Arabidopsis thaliana*.

Design requirements for intragenic vectors (Conner et al. 2007)

Whole plant derived vectors / intragenic vectors must be entirely derived from plant DNA sequences and must contain the following elements:

- Homologous region corresponding to a bacterial origin of replication
- Plant genomic sequences similar to the shortest bacterial origin of replication (ColE) have been found in a wide variety of plant species. These plant derived fragments can be assembled into a vector containing plant derived T-DNA elements (Barrell et al. 2010).
- Selection marker for maintaining the plasmid in bacteria.
- The smallest bacterial selection marker is based on a short lac-operator sequence which is part of the operator repression titration system of *E. coli*. Lac-operator like sequences have been found in a wide range of plant species. These plant derived sequences can be assembled into a vector containing plant derived T-DNA elements (Barrell et al. 2010).

- Intragenic vector elements should fulfil the following requirements:
- Plant DNA fragments for simulating the bacterial T-DNA should not contain regulatory (e.g. promoter) sequences
- Plant DNA sequences for T-DNA generation should not be retrieved from heterochromatic chromosomal regions
- A significant stretch of plant DNA should be added outside the left T-DNA border
- The intragenic vector should comprise of a minimal number of genetic elements: this design would mimic naturally occurring DNA rearrangements in plant genomes

- Plant endogenous genes conferring resistance to herbicides or antibiotics should be preferred.
- Selectable marker genes can be avoided in plants that take up DNA with high efficiency (e.g. potato).

9.4 Marker-free technologies - Selectable markers and marker elimination methods

(If not otherwise stated the information is retrieved from König 2003)

Marker genes in transgenic crops are a major issue of concern. However, given the usually low transformation rates, in most cases they are necessary to select for rare transformed cells.

The amount of novel recombinant DNA in transgenic organisms should be limited to an absolute minimum (Kuiper et al. 2001), which is also underlined by policy makers in the United Kingdom, the European Commission and the United Nations Food and Agricultural Organisation/World Health Organisation (König et al. 2004). The application of marker excision technology is encouraged (Rommens 2009).

Selection markers (for a comprehensive review see Miki and McHugh (2004))

For the choice of an appropriate selection marker basically two options are available:

- cytotoxic (= negative) selection markers, and
- metabolic (= positive) markers

Negative selection markers – antibiotic resistance genes

Negative selection markers mediate resistance to cytotoxins, which either inhibit growth of non-transformed cells or kill plant cells directly (Darbani et al. 2007). At present, selectable markers are employed which usually confer resistance to antibiotics or herbicides (Woo et al. 2011).

The most abundantly used selection marker system is based upon the bacterial neomycin phosphotransferase II (*npt*II*)* gene conferring resistance to kanamycin, neomycin and geneticin (Darbani et al. 2007).

The EFSA GMO Panel released an Opinion on the use of antibiotic resistance genes in GM plants in 2004 providing a 3-tiered classification scheme for antibiotic resistance genes (category 1: no risk for human and animal health and the environment; category 2: should only be used for authorized field trials; category 3: should not be used in transgenic crops in general). The Panel

concluded that the use of *npt*II as a selection marker did not pose a risk to the environment or to human and animal health (EFSA 2004). EFSA provided two additional statements including a "Joint Scientific Opinion of the GMO and BIOHAZ Panels" on the use of ARM genes in GM plants in 2009, which resulted in the same conclusions (EFSA 2007; EFSA 2009). However, two Members of the BIOHAZ Panel expressed a critical minority opinion. Additionally this Joint Opinion pointed to limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source and stressed the importance of taking those and other uncertainties described in that Opinion into account (EFSA 2009).

In general, the European Commission discourages the use of antibiotic resistance genes as selection markers in transgenic plants for commercial purposes. Moreover, according to Art. 4 (2) of Directive 2001/18/EC "Member States and the Commission shall ensure that GMOs which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment, with a view to identifying and phasing out antibiotic resistance markers in GMOs which may have adverse effects on human health and the environment. This phasing out shall take place by the 31 December 2004 in the case of GMOs placed on the market according to Part C and by 31 December 2008 in the case of GMOs authorised under part B".

Negative selection markers – herbicide tolerance

In maize the application of the glyphosate tolerance system is less efficient than the utilisation of the *npt*II marker technology. However, herbicide tolerance is a mature technology (König 2003; Darbani et al. 2007).

Positive metabolic markers

Positive selection markers allow transformed plant cells to grow on exotic growth media (i.e. unusual carbon sources) that do not support the growth of unmodified cells, or produce precursors for growth hormones that allow only the growth of transformed cells (Wei et al. 2012). The most important representative of this marker class and closest to marketing is the enzyme phosphomannose isomerase (PMI) (Stoykova and Stoeva-Popova 2011). Transformation frequencies are ten times higher compared to the *npt*II system in maize. However, this methodology cannot be regarded as mature because successful transformation protocols are only available for a limited number of plant species (Wei et al. 2012). Accessibility is restricted via patenting and the Interference with the normal plant metabolism, resulting in pleiotropic effects, may be envisioned. There might be potential adverse effects on the ecosystem

due to plants which show a phenotypic growth advantage, compared to their wild type counter parts (König 2003).

However, safety analyses of the PMI system revealed no toxic or allergenic effects of the protein. There were no apparent changes in the glycoprotein profiles of transgenic maize and sugar beets and field trials showed no differences in agronomic performance or grain composition between GM maize and non-GM controls. Transformation rates with the PMI system were ten times more efficient compared to the *npt*II selection system in sugar beets. These observations are indicative for a certain advantage of positive metabolic marker systems concerning hazard and risk assessment (Miki and McHugh 2004).

9.4.1 Selectable marker elimination

Marker elimination methods provide, besides an increased regulatory and market acceptance, additional advantages for a repeated transformation of the same cell line, allowing the introduction of multiple transgenic traits by using the same marker gene (Hare and Chua 2002).
For the removal of antibiotic or herbicide resistance marker genes the following three methodologies may be applied:

- co-transformation
- excision by homologous recombination
- recombinase-induced excision

Co-transformation

Usually, the marker gene is placed on a second T-DNA containing vector, which may be physically segregated and lost in the subsequent generations of the plant (see Figure 7b) (Woo et al. 2011).

Modified protocols for plant cell transformation with *Agrobacterium* result in the integration of the selectable marker and the transgene into two different genomic loci, preferentially into two different chromosomes. This constellation allows segregation of the two loci by breeding, resulting in a cell line which has lost the resistance marker, but still contains the desired transgene (Woo et al. 2011). The technology is considered mature and efficient (up to 25% of all co-transformed cell lines show marker segregation), but screening becomes more tedious and costly because four times more cell lines have to be checked. Patents restrict accessibility to this technology. Functionally irrelevant marker gene sequences are clearly separated from the new transgenic trait, and do not appear anymore in the adult plant (König 2003).

Homologous recombination – recombinase induced excision

Due to homologous recombination between identical DNA sequences flanking a central region, this central DNA fragment can be excised (Woo et al. 2011). Despite some initial success, the naturally occurring recombination frequencies in plants are rather disappointing and the whole process is difficult to control. The technique, at this stage, is not yet considered mature but may be promising for future applications (König 2003).

Loop-out recombination in plastids (Klaus et al. 2004)

This approach was successfully applied with tobacco, tomatoes and potatoes. Mutant plants were transformed with constructs containing intact elements of the marker genes and the gene of interest surrounded by flanking sequences prone for homologous recombination. After release of the selection pressure the marker gene is excised by loop-out-recombination leading to marker free plastome transformants containing only the gene of interest (see Figure 7e) (Rommens 2004; Conner et al. 2007).

Recombinase induced excision

The recombinase enzyme cuts two short DNA recognition sequences and ligates the free ends after the elimination of the DNA sequence positioned in between. The Cre/lox system from bacteriophage P1 is predominantly used in plants relying on three different strategies (Zuo et al. 2001):

- Autoexcision
- Transient transfer
- Outbreeding with Cre carrying plant cell line

Autoexcision

The marker gene flanked by two lox-sequences is co-transformed with the *cre* gene. The expression of the *cre* gene is regulated either tissue-specific, growth phase dependent or by an inducible promoter (see Figure 7). This is a rather new technology and complicated during execution. The transformation efficiency is too low for commercial application. Patents limit accessibility. Moreover, it is not clear which methods should be applied for the risk assessment because Cre/lox may induce unintended recombinations within the plant genome. These unsolved problems may support opponents of the technology (König 2003).

Transient transfer

Transiently the CRE-Protein is directly transferred into the targeted plant cell via microinjection. Strains of *Agrobacterium* carrying two different T-DNA containing vectors harbour one vector carrying a positive and a negative selection marker gene whereas the other vector contains the gene of interest. After transformation a transient positive selection step is followed by a negative selection for marker gene integration. This approach leads to approx. 29% of plants containing the desired DNA sequences (see Figure 7d) (Rommens 2004).

Outbreeding with Cre carrying plant cell line

Generation F1 plants carry both the *cre*-sequences and the transgene. In generation F2, out-crossing will eliminate the *cre*-sequences. A single *lox* insertion site and the transgene will remain in the plant genome (see Figure 7a) (Rommens 2004). This technology is mature and can be applied in many plants of commercial interest. However, it is certainly more resource consuming and less cost efficient than the *npt*II marker gene system (König 2003).

Marker-free transformation

Using supervirulent strains of *Agrobacterium*, transformation frequencies can be substantially raised with certain plant species (e.g. up to 5% in potato) which may generate a sufficient number of transformants eliminating the need for a selection step (see Figure 7c) (Rommens 2004).

Effects of the introduction of selectable marker elimination methods

In the following paragraphs, positive and negative aspects of an implementation of selectable marker elimination methods will be discussed (König 2003; König et al. 2004; Rommens 2009).

The major advantages of this new technology basically would be the elimination of gene sequences irrelevant for the adult plant or organism, the minimisation of the input of foreign recombinant DNA into the plant genome, the possibility to re-transform the same transgenic cell line with the same marker gene for the introduction of additional desired transgenic traits, reduced occurrence of gene silencing, which may take place if one or more identical regulatory or coding regions are present in the targeted genome. The first two issues would ease risk assessment. The last issues cause experimental and technical concerns because they would substantially increase the possibilities for manipulation of plant cell lines (König 2003).

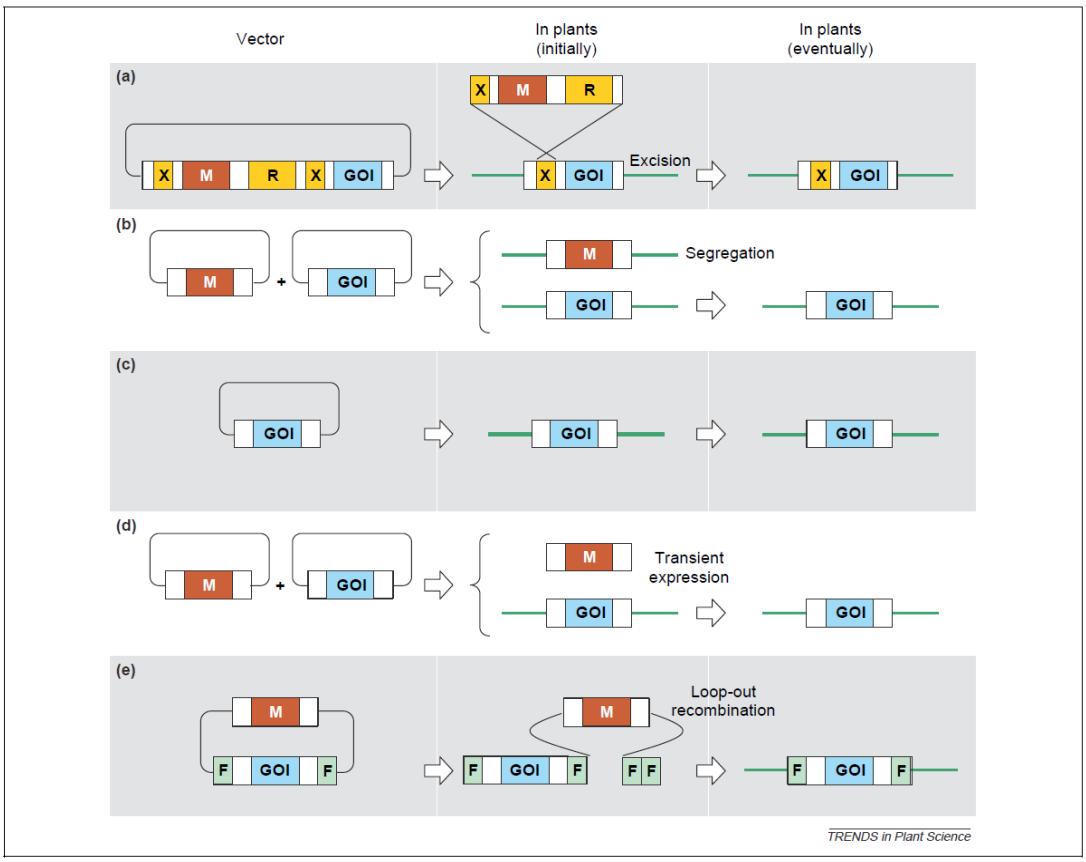


Figure 7. Marker excision procedures (from Rommens 2004).
(a) An expression cassette for a selectable marker gene (M) is placed, together with an expression cassette for a recombinase (R), between recombination sites (X). Upon transformation, expression of the recombinase gene might result in excision of the region between the recombination sites, thus enabling the recovery of transgenic plants only containing an expression cassette for the gene of interest (GOI) and a single remaining recombination site. (b) Co-transformation of plants with both a T-DNA vector carrying the selectable marker gene and a vector containing the GOI might result in unlinked integration events, which can be segregated in progeny plants. (c) T-DNA lacking can be used to generate transformed plants infrequently. (d) Co-transfer of two T-DNAs, one carrying M and the other containing the GOI, followed by a transient selection step results in the frequent integration of only the GOI T-DNA. (e) Plastid transformation using a vector in which M is cloned outside the homologous flanks. The marker co-integrates via a first recombination but is excised upon a subsequent loop-out recombination event.

The major disadvantages of this new technology basically would be the cost-efficiency of the presented alternative technologies compared to the established systems (=*npt*II); novel, intrinsic risks, which may have effects on regulatory issues and approval; lack of maturity of the most promising method (autoexcision), which still cannot be applied to a wide variety of different plant species and restricted access to these new technologies (patents).

Technology assessment in this context is complicated by the rapid scientific progress in the field and changes in societal norms and values. The assessment process, therefore, must be dynamic, iterative and recursive, and must be adapted to new realities over time (König 2003).

10 Detectability of modifications in plant genomes

10.1 Methods of GMO testing – state-of-the-art

Genetically modified organisms (GMO) have been engineered through the stable integration of a recombinant genetic cassette into the genome of a recipient organism. The purpose of generating such a novel organism is to provide the new variety with specific features. The diversity and number of GMOs which get into the food chain or the environment, intended or unintended, is rapidly growing, so the possibility to detect and characterize GMOs is fundamental. Information on the DNA-modification is available for plants falling under GMO-regulations, so consequently the process of verifying these modifications is not the challenge.

Without any information concerning the potential alteration of a plant genome the detection is hampered, similar to that of unauthorized GMOs, falling under the European Network of GMO Laboratories (ENGL) Classification level 4 (unknown GMOs; construct consists of only unknown elements; ENGL 2011). A detection of those elements is not possible because of the lack of information about the used modification and as a consequence of the lacking authorisation-process by EFSA, no official detection method is available. Also with the existing already validated methods those novel modification cannot be detected.

Most frequently applied tools for GMO-testing are bioassays, protein-based assays and DNA-based assays (Holst-Jensen 2009). However, also other technologies have been developed, e.g. computational subtraction (Tengs 2009), and may be implemented in the future.

The choice of the appropriate method for a given sample depends on its kind and constitution, whether single traits or multiple events should be detected, or whether the identification and/or quantification of a genetic modification is required, the methods may be used individually or may be combined to achieve the desired results.

10.1.1 Bioassays

Bioassays are based on the principle of exposing plant seedlings from a certain seed batch to a specific substance, e.g. an herbicide. Resulting from the specific trait the seedlings harbour, the genetically modified (GM) kernels are tolerant

whereas non-GM-plants are not. To determine the relative GMO content in the seed lot, the number of kernels which are resistant to the substance is compared to that of sensitive. The advantages of bioassays are low costs, few requirements for user competence and the possibility to confirm the desired biological traits of the GM plant. Such trait can be a drawback when other traits should be confirmed, too. In comparison to DNA-based assays the specificity is limited and the procedure is time-consuming.

10.1.2 Protein-based methods

Immunological and physicochemical techniques are applied to detect proteins resulting from the expression of specific genes. The most common protein-based assays are immunoassays. Here, the target proteins act as antigens, which are detected by specific antibodies coupled to a colorimetric detection system. This is the principle of the laboratory based enzymatic reaction called ELIZA (enzyme linked immunosorbent assay). An alternative would be portable LFS (lateral flow strips) which can be applied in the fields or at storage- as well as processing facilities. Nevertheless relatively high costs of developing specific antibodies as well as the low power of quantification, caused by a potential weak expression and translation of genes, account for the limited routine use of protein-based assays. Quantitative application is possible under certain conditions but is usually no adequate method for highly processed or composite products.
Alternatively, protein-detection can be done by means of mass spectroscopy or 2-dimensional gel electrophoresis.

10.1.3 DNA-based methods

Since the genetic modification is accomplished on the DNA itself, DNA-based methods are at the highest level of metrological traceability compared to methods that detect products thereof. DNA-based assays display higher specificity and sensitivity in comparison to protein-based ones. Their drawbacks are primarily high costs and advanced competence requirements.
Measurement of DNA by PCR (polymerase chain reaction) has been widely used when detecting GMOs. In addition to conventional gel electrophoresis, a wide range of possibilities for detection and identification of PCR-amplified targets exist, like the use of capillary gel electrophoresis, hybridization to labelled and coloured beads, and flow cytometry.

Real-time PCR is currently the method of choice, as it also allows for the quantification – for which it has been developed – of GMOs. In general, the testing strategies followed by laboratories consist of two phases. First, GMO

presence is detected by a screening, which is carried out in order to detect common target-elements in GM crops. If GMO-presence is confirmed, an identification of GMOs is performed using event-specific methods. A range of event-specific methods submitted by applicants for authorisation is validated and tested in the EU by the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF, formerly named Community Reference Laboratory; Aguilera et al. 2009).

When several signals indicate the presence of more than one event, it is not possible with current methods to distinguish whether the signals are caused by the presence of individual GM lines or by a line harbouring stacked events.

For the detection of UGM (Unauthorized Genetic Modifications) there are currently no methods that reliably detect potential unknown modifications if the alteration of the modification is unknown (Tengs 2009). A possibility to get an idea of unknown events may be the application of the so-called matrix-approach (Ruttink 2009). This method is based on the comparison of results of the screening with those of the identification. Successful identification of the unknown event is not possible. However, screening indicates that the organism contains foreign DNA-elements. Hence, the matrix approach does not provide conclusive evidence of the presence of unauthorized GMOs.

10.2 Detection of cisgenic modifications

Whether a cisgenic organism will be detectable or not depends on the following issues (Sustainability Council of New Zealand 2011):

- the nature of the genetic modification, including the genetic material inserted
- the availability of detection methods
- whether the regulator obtains information or methods that allow for detection (depends on the classification of the organism – non-GM/GM).

Following the definition of “cisgenesis” it is of utmost importance to verify the cisgenic nature of a given plant. To achieve this, sequencing of a large stretch of the flanking region including the adjacent plant genomic sequence is a straight-forward way. The definition of cisgenic plants implies that no foreign sequences deriving from agrobacteria or vectors used in the transformation process are present. The insertion of *Agrobacterium*-specific sequences in plants has been reported (Ulker et al. 2008).

Cisgenic modifications of plants may result in products/seed etc., in which the detection of the modification is hampered by the nature of the modification, e.g. the insertion of additional copies of genes already present in the plant

genome. Whereas cisgenic modifications can be clearly detected on the DNA-level, provided that the relevant information is available, the use of protein-based methods for the same purpose is heavily restricted. In many cases, an inserted gene from a crossable species leads to the expression of a protein that is highly similar to those already expressed in the plant. Alternatively, additional copies of an endogenous gene may be introduced, resulting in elevated levels of the protein encoded by this gene. In contrast, detection of the newly expressed protein is possible via protein-based methods if an inserted gene leads to the expression of a new protein previously not present in the plant.

In order to reliably detect cisgenic modifications in plants using currently available methods in routine analysis, information on the alteration, i.e. the cisgene itself, as well as on the flanking regions of its insertion site has to be available to the testing laboratory. In the EU, to date GMO screening for enforcement purposes is mainly performed with methods for detecting DNA sequences and applying PCR technology (Real Time PCR; JRC 2011). For this, the above-mentioned information (inserted genetic material, its arrangement, and in particular the unique flanking sequences of the insertion site) is necessary to develop an event-specific method for detection. As foreign DNA sequences are absent it is not possible to employ general screening methods. Furthermore, cisgenic organisms may be distinguished from their conventionally bred counterparts only based on the insertion site of the cisgene. Due to the currently available plant transformation methods the integration occurs randomly and therefore results in a specific event with unique flanking sequences. Consequently, the unequivocal detection and identification of a cisgenic modification is possible.

An additional possibility to detect and trace back cisgenic modifications would be to modify plants introducing unique detection markers along with the cisgenic DNA sequence (Sustainability Council of New Zealand 2011). Such an approach could facilitate routine analysis and may in addition make screening possible.

How easily cisgenic plants will be captured by existing detection methods remains the subject of speculation as no cisgenic product has made it to the market (Sustainability Council of New Zealand 2011). A crucial question will be whether a cisgenic modification can be detected in routine control-analysis. Here, the questions to be answered are similar to those approached in so-called blind tests. First, “modification - yes or no?” and second “which kind of modification?”; in routine control, both questions have to be answered unequivocally.

Only unambiguous detectability allows the use of GMOs during the food production chain and therefore gives the consumer the possibility to make a

qualified choice. Detection of cisgenic GMOs may be confounded by a lack of suitable methods (a technical deficiency) and/or constraints on what information regulators can require (a regulatory deficiency) (Sustainability Council of New Zealand 2011). Regulatory requirements play an important role in determining whether cisgenic organisms are readily identifiable and traceable (Regulation (EC) 1830/2003, Directive 2001/18/EC). As part of the EU's traceability and labelling requirements for post-marketing surveillance, site-specific sequence data for the entire inserted DNA, along with adjacent genomic sequences near the insertion site, are required for event-specific tracking purposes.

10.3 Detection of small alterations in the genome (e.g. ODM, ZFN-1)

Mutations may, for instance, be induced by radiation (UV, radioactive) or by the long-term application of pesticides. Also, mutation breeding has been applied for many decades, resulting in plants with different small modifications in their genomes. Small genomic modifications may not be noticed (silent mutations) as they do not necessarily result in neither phenotypic nor obvious physiological changes. Such unintended changes may also result in beneficial traits, rendering the plant more competitive in its environment.

Modifications of the entire genome in plants, like mutations, deletions or insertions, are commonly detectable by the current molecular biological methods that focus on DNA-based analysis. As the observed mutations are always directly linked to changes in DNA sequences, the straightforward detection with specific methods, e.g. High Resolution Melting (HRM) or Restriction Fragment Length Polymorphism (RFLP) is possible. Genetic screening methods are used to identify sequence variation in (plant) genes. Mutants may be identified using diverse molecular techniques, like DNA fingerprinting and mapping on PCR based markers, such as RAPD (Rapid Amplified Polymorphic DNA), ALFP (Amplified Fragment Length Polymorphism), or STMS (Sequence-Tagged Microsatellite Sites) (Ahloowalia and Maluszynski 2001). Also, sequencing may be used for DNA-based mutation detection. Today, Targeting Induced Local Lesions in Genomes (TILLING) and Ecotilling, respectively, are among the most widely used methods (Colbert et al. 2001; Till et al. 2003; Comai et al. 2004; Kurowska et al. 2011). Whereas TILLING includes the induction of mutants, Ecotilling describes the identification of natural DNA variation. Additionally, mutation/polymorphism screening techniques based on massive parallel sequencing have been commercialized (Rigola et al. 2009). The approach allows high-throughput mutation detection in early generations of mutagenized and natural populations.

Given the precondition that plants are subject to GMO regulations, information on the DNA-modification is available. The prior knowledge on the modification is the key factor for rendering its detection possible. In this case, unmodified plants may clearly be distinguished from modified ones. Also unintentional mutations, e.g. as a result of evolution or as a side-product of intended modifications of the plant genome, can be detected, at least by direct sequencing. Although the detection of small mutations in the genome is possible with current methods, their identification, i.e. the clear verification of their origin, is not. Generally, they cannot be distinguished from those occurring spontaneously.

For plants produced by ODM and ZFN that harbour point mutations a clear additional challenge is to identify the source of the genetic modification. It is essential to distinguish between detection and identification: **Detecting a change** means the possibility of determining the existence of a change in the genome in comparison to conventional counterparts or natural variants. The **identification of a change** implies the possibility of determining that this particular change in the DNA has been intentionally introduced by targeted mutation techniques, e.g. ODM or ZFN.

10.4 Detection of stable and transient *Agrobacterium*-mediated modifications

Like for all GM methods leading to stable integration of additional sequences, detection of stable genomic alterations caused by *Agrobacterium*-mediated transformation is possible by means of current standard molecular detection methods (Real Time PCR) if adequate information on the alteration is available. In addition, the newly expressed protein may be detected by protein-based methods, as both primary transformants and their progeny express the desired product.

In agroinfiltrated/agroinoculated plants the introduced genes are expressed transiently (starting a few days after transfer of the constructs and lasting up to 3 – 5 days (Choi et al. 2011) to 5 – 7 days (Baskhar et al. 2009). The constructs are not integrated into the genome. Detection on protein-level or by visual marker techniques is limited to a very short period of time. During the expression of the novel protein, its detection is possible with protein-based methods. Successful infiltration leading to the expression of the desired protein may also be visualized by additional visual marker techniques like photo-bleaching (Bazzini et al. 2007).

As stable integration and thus changes in the genome are not foreseen, detection of an agroinfiltrated plant will as a matter of fact not be possible on DNA-level. It is generally presumed that the genome of an agroinfiltrated plant

is free from any *Agrobacterium*-sequences and from vector sequences resulting from the transformation process itself. Thus, no foreign DNA-sequences are expected to be found in the host plant genome or in the progeny. Prior to further propagation of plant tissue infected with *Agrobacterium* the plant material should be screened for the absence of any foreign DNA sequences, e.g. chromosomal *Agrobacterium*-DNA or the T-DNA used for the agroinfiltration. If free from foreign DNA, an organism resulting from agroinfiltration is not distinguishable from its conventional (wild type) counterparts on the DNA level.

Following some important considerations, in general the unintentional release of *Agrobacterium*-cells is not expected to happen due to limited handling time. The absence of agrobacteria on/in infiltrated plant tissues should, however, be verified. Weller et al. (2002) detected (wild type) agrobacteria in *Brassica napus* seeds, which indicates that survival of agrobacteria inside seeds cannot be excluded. Plant material (leaves, seeds, roots) may be analysed with effective and sensitive detection methods such as PCR or quantitative PCR (Cubero et al. 1999). Cubero et al. (2006) used a methodology based on a combination of (bacterial) isolation methods and PCR technology for detecting agrobacteria in plant tissue. Similar to the detection of agrobacteria, *Agrobacterium*-derived sequences can be detected using PCR-based methods. Using this method, the applied transformation technique may be indicated. Due to the potential survival of agrobacteria within infiltrated plants, they may potentially lead to the integration of foreign sequences and leave characteristic footprints in the genome. Potentially, this may also happen in techniques aiming at transient expression.

10.5 Future developments in routine testing for GMO

Current methods are generally expensive and time-consuming and mostly carried out in the laboratory (Holst-Jensen 2009). It is expected that tests, particularly for known GMO, will become increasingly faster, affordable and portable for on-site use throughout the supply chain – on the farm as well as during processing and, of course, in the laboratory (Sustainability Council of New Zealand 2011). They should be applicable for both multiplexing and quantification.

Novel transformation technologies may redefine the types of analytical target. Cisgenic or intragenic modifications frequently exclude the use of protein-based detection methods as the gene and thus the protein may already be present in the plant. Possible alternatives include DNA-sequencing approaches, or mass spectrometry.
An analytic approach for the detection of transgenic/cisgenic/foreign sequence elements is their fingerprinting by using a genome-walking type PCR. This approach can provide information on the plant genome that flanks the transgenic/foreign sequence (Raymond 2009). A DNA-fingerprint (made by specific restriction enzymes) is characteristic for a known GMO-event. A new pattern will indicate the presence of new/foreign sequences different from known GMO-constructs and events.

International harmonization and exchange of information concerning GMO-developments among competent authorities und the associated control laboratories may also facilitate the monitoring of unauthorized GMOs.

A relatively new strategy for identification of foreign sequences is called computational subtraction (Tengs 2009). Assuming that the whole genome-sequences of most plants are already identified and the costs of sequencing will further decrease, high-throughput-sequencing will be employed to obtain the DNA or RNA sequence data from the sample. Using sequence similarity search algorithms, the data is compared against a set of reference sequences from a generated database. All the endogenous (expected) reads will be filtered away leaving only a small collection of sequences that appear not to originate from the sample organism.

Currently, the routine detection of cisgenic modifications requires molecular information on the event, which will be necessary to allow for its detection in the test laboratory. All other methods would be too costly and laborious.

11 Traceability

11.1 Traceability in seed

Products produced from GM varieties are subject to the national/European regulations for GMO, concerning seed in particular the Austrian Ordinance on GM-seed 2001 (BGBl. II Nr. 478/2001). Seed may only be produced, certified, and placed on the market if the variety is registered in the common catalogues of varieties (Council Directive 2002/53/EC and Council Directive 2002/55/EC). GM varieties are clearly identified in the common catalogues by means of footnotes indicating the EU authorisation of an event with the relevant Commission Decision.

The bases of the common catalogues are the national variety lists of the member states. The registration procedure for the national variety lists stands at the end of the variety development process, independent whether a new genotype is GM or non-GM. In case of a GM variety, all regulatory requirements foreseen on EU and national level apply. Thus, before using an event for the development of a new variety, the event has to be approved by the European Commission. At the very beginning of the variety registration process, the applicant has to provide a written confirmation whether the variety is GM or non-GM. In addition to the usual traceability system during seed production, the GM status of a variety is documented throughout the seed production process. Products produced from a GM variety (according to the respective footnote in the common catalogues) are subject to the national/European regulatory framework for GMOs.

Generally, when applying for variety registration the applicant has to give information about the genetic background (crossing partners, gene pool for selections, etc.), pedigree and breeding methods used in developing the new genotypes (variety candidates) in a crop-specific technical questionnaire. The breeder may also inform about special methods applied during the breeding process, e.g. doubled haploid (DH)-methods, protoplast fusion, or mutation breeding. In the current system, due to intellectual property (IP) protection, only limited exact information may be available. During the variety registration procedure, the candidates are thoroughly described according to international standards (e.g. UPOV guidelines), which is the basis for identification of a variety.

Seed significantly determines the amount, quality and safety of harvested crops for producing feed and food, and is subject to detailed regulations worldwide to ensure that extensive precautionary measures are taken to secure food and raw material safety. Due to the strategic importance of seed for the economy,

Europe, the OECD countries and many other countries in the world prefer applying pre-marketing product certification to subsequent control.

The certification system for seed encompasses a quality system that is suited to observe the genetic identity and varietal purity over several generations, starting from breeder's seed to pre-basic or basic seed up to the certified seed. Checkpoints and system monitoring with regard to good agricultural practice as well as good practice in seed processing, traceability including their examination and monitoring are the subject of the quality system "seed certification". The seed certification system covers precautionary measures, the implementation of technical standards for basic material, the farm, the seed production area and harvesting, transportation, storage and processing measures, including the bagging, sealing and labelling of the certified seed product. The Austrian Ordinance on GM-seed 2001 (BGBl. II Nr. 478/2001) foresees measures to avoid the presence of GMO in seed produced and/or marketed in Austria.

Seed certification procedure

In seed production, traceability due to the requirements of the European seed certification system is given. Generally, traceability is ensured through unique lot identity numbers and defined duties to keep records throughout the seed production, processing and distribution processes. If seed is produced from a GM variety, the clear labelling of the seed as GM is mandatory. Control relevant documentation about suppliers and customers has to be kept within the whole seed certification system according to §9, Austrian Seed Act 1997 as amended (BGBl. I Nr.72/1997). An example of the questionnaire applied for the relevant audits is given in the annex (part 2).

Any company/organisation producing, bagging or processing seed has to be registered by the designated seed certification authority. They are legally obliged to keep records about, e.g., the quantity and identity of seed, as well as the name and address of the primary recipient, but also on the disposition of seed not fulfilling the standards for certification.

1. Contracting and acquisition of basic seed:

   The farmers (seed producers) close private contracts with companies/organisations intending to produce seed (applicant for seed certification). For field production, the applicant has to provide the farmers with certified pre-basic or certified basic seed with unique lot numbers. After sowing, the farmers keep the labels and proof of legal acquisition of the seed. In case of hybrid varieties the maintainer of the variety gives his written consent to produce seed of the given variety and defines the parental lines to produce the F1.

2. Application for Seed Certification:

   The legal requirements define a set of rules concerning the application and duties of the applicant of seed certification. The certified pre-basic or basic seed in case of application for seed certification has to be lawfully acquired (Austrian Parliament 1997, § 10). The application received by the designated authority contains defined data, e.g. information concerning name and address of applicant and seed producer, species, variety, category of produced seed, localisation and size of production field, year of harvest. In addition, the variety has to be listed in the Austrian list of varieties, if this is not the case its valid registration in a Member State or in the EU Common Catalogue has to be assured. In principle, this applies to both GM and non-GM varieties. However, national bans preclude the growing of GM varieties in Austria.

In each official procedure according to Austrian Seed Act 1997 (BGBl. I Nr.72/1997) the applicant has to provide a written confirmation to comply with the requirements of the Ordinance on GM-Seed 2001 (BGBl. II Nr. 478/2001, §4). Concurrently, a GM variety or seed of a GM variety has to be clearly labelled as “genetically modified variety” or “genetically modified seed” including the identity of the GM event.

The designated authority carries out all technical examinations in field inspection and seed testing.

3. Field inspection:

Field inspection, *i.e.* the observation of varietal identity according to the variety description as well as adequate maintenance of the seed production field, is done by official inspectors and authorised inspectors under official supervision. The results of the field inspection are documented in separate and defined forms containing, among species- and category-dependent information, the unique field identity number. If either varietal purity or varietal identity does not conform to the varietal description the field is excluded from further seed production. Immediate actions are necessary in case of “off-types” (potential GM admixture), which will be roughed well before flowering. Fields complying with all legally required technical and traceability standards will be approved and further used for seed production.

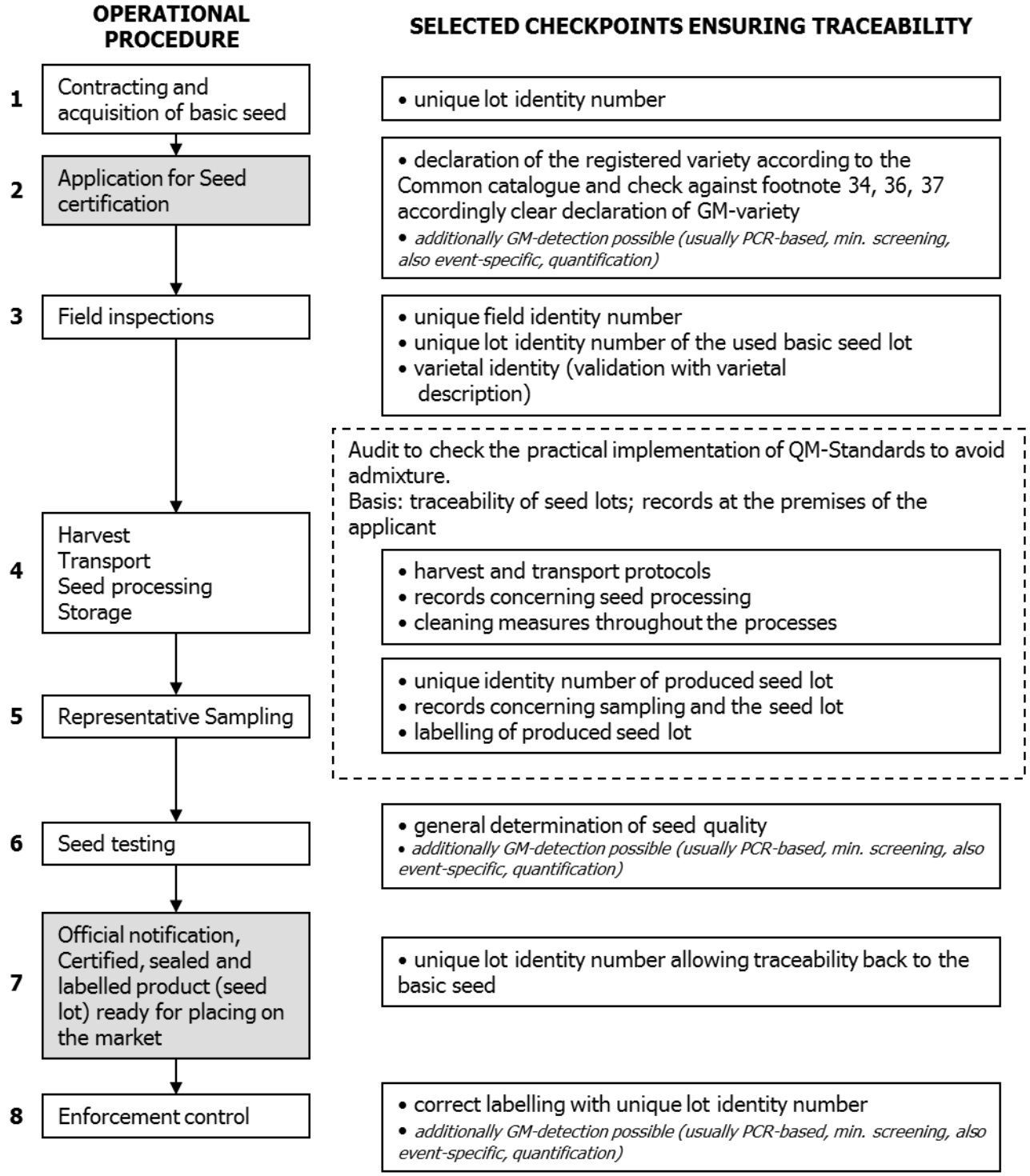
4. Harvest, transport, seed processing, storage:

Seed production fields are either harvested separately or, if of the same variety, may be harvested and transported together. In any case, the identity of the seed during harvest and transport is documented by the applicant. The accurate cleaning of machinery (including combines and transporters) has to be verifiably confirmed.

At the site of takeover the unique field identity number is again checked and a unique lot identity number is assigned. The latter is used throughout all following processes, *i.e.* storage, processing, treatment, sampling up to the bagging and labelling of the final product. The admixture with seeds of other varieties or species is avoided by accurate cleaning of the processing machinery. The full documentation of the traceability, all cleaning measures and the labelling is supervised by the designated seed certification authority.

Traceability in seed production, processing and distribution

The unique lot identity number allows traceability from the certified product back to the basic seed



Footnote 34: Genetically modified variety. Marketing of seed permitted under the conditions laid down in Commission Decision 98/294/EC.

Footnote 36: Genetically modified variety. Marketing permitted under the conditions laid down in Commission Decision 2010/135/EU.

Footnote 37: Genetically modified variety. Marketing of seed permitted under the conditions laid down in Commission Decision 98/293/EC.

Figure 8. Traceability and process control in seed production

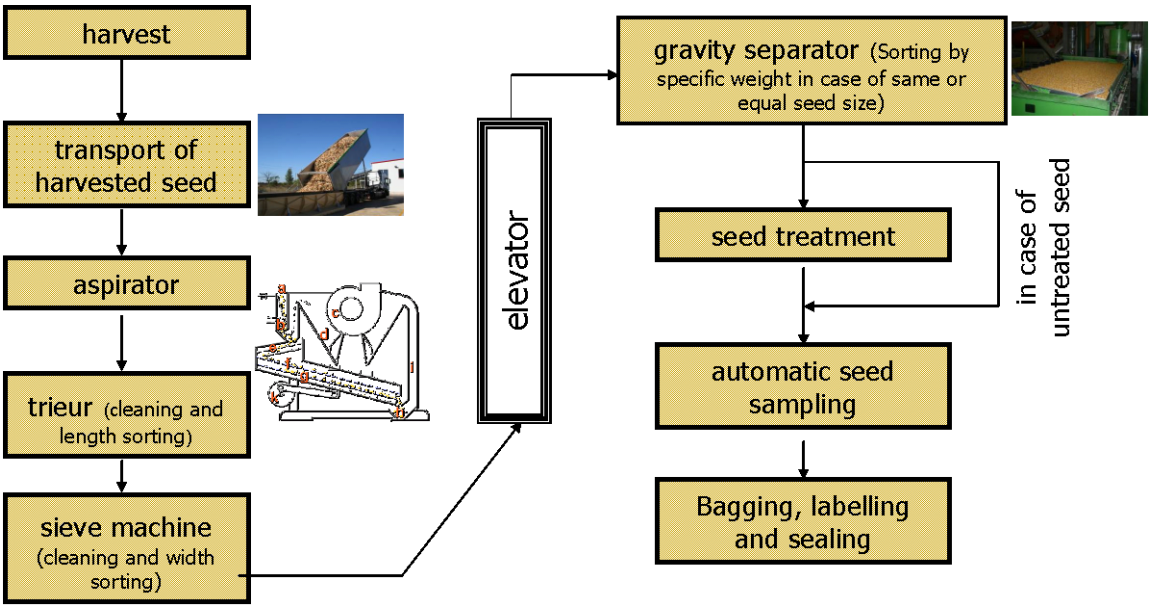


Figure 9. Simplified view of seed production procedure in the seed plant

The processed seed has to be clearly labelled - including the unique seed lot identity number - on the exterior of the seed bag (Austrian Parliament 1997, §15). Furthermore the label has to contain, *inter alia*: information on the species, incl. the botanical name, variety name, seed category, unique lot identity number, information on the treatment. The colour, shape and size of labels follow legal specifications.

Seed of authorised GM varieties have to comply with the regulations in the Ordinance on GM-seed 2001 (BGBl. II Nr. 478/2001). In all seed categories, such seed has to be clearly labelled as “genetically modified variety” or “genetically modified seed” including the GM line and the trait; the genetic modification also has to be indicated on all shipping documents (BGBl. II Nr. 478/2001, §5). Like all varieties used for seed production, also GM varieties have to be published in the list of varieties and therein clearly identified as “genetically modified variety”. The Ordinance on GM-seed 2001 (BGBl. II Nr. 478/2001) also extends the requirement to identify a variety as GM to sales catalogues and advertising materials.

5. Representative Sampling:

During the seed certification procedure a representative sample to determine the quality of the seed to be certified is mandatory. Sampling is based on national and international regulations. Different procedures of sampling are possible (see Figure 10). In case of automatic sampling from a seed stream a closed and sealed system is obligatory. Automatic sampling is supervised by the seed certification authority. Authorised or official seed samplers are responsible for manual sampling. In both variants traceability

is ensured through the unique seed lot identity number. Seed sampling results in a sample representative for the whole seed lot (representative sample), from which the working sample for seed testing, including GM detection, will be taken.

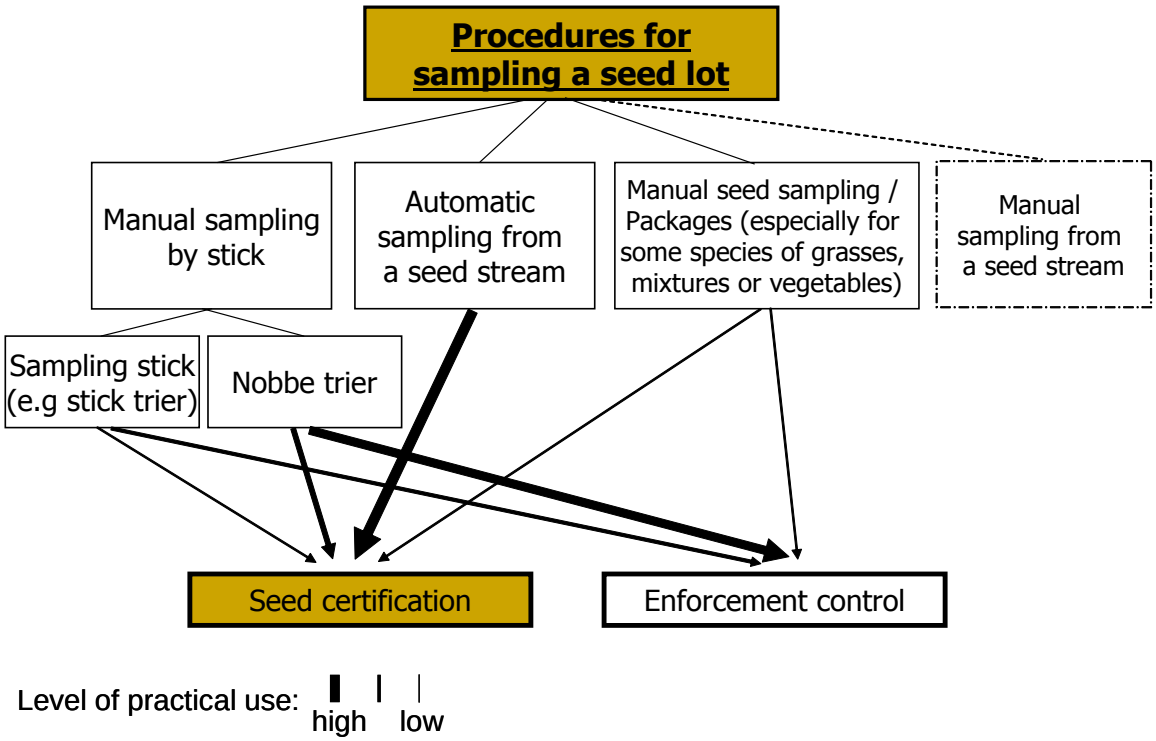


Figure 10. Sampling procedures

6. Seed testing:

   Different parameters are checked to ensure appropriate quality of a seed lot to be certified, *inter alia* the health status, seed purity, germination rates plus the detection of GM impurities to verify the GM status.

7. Official notification:

   A positive certificate is the requirement for placing seed on the market. The certificate is issued by the designated seed certification authority based on the fulfilment of minimum quality standards as laid down in national and international regulations. The certified product is then used by farmers to produce food and feed. Through the unique lot number on the label and on the official notification the whole production process is reliably documented in all its steps, and the traceability from the final, certified product back to the basic seed is guaranteed.

8. Enforcement control:

   Independently from the seed certification process, labelling and sealing of seed placed on the market is controlled by official inspectors of the seed certification authority. In addition, samples are taken from the seed lots according to a risk-based, integrated control plan and are checked for variety identity and seed quality, including the GM status. In case of any non-compliance sanctions are imposed.

11.2 Traceability in food and feed

According to EU legislation, informative traceability systems have to be established by the Member States with the aim to protect "human health and consumers' interest in relation to food" (according to Article 1 of Regulation (EC) No 178/2002). Based on these regulations, common principles and responsibilities as well as efficient organisational arrangements and procedures are ensured throughout the EU. Specific legislative measures have been set into force concerning the traceability and labelling of GMOs. Recital 4 of Regulation (EC) No 1830/2003 explicitly aims at the "freedom of choice" of consumers, similar to Recital 17 of Regulation (EC) No 1829/2003 which states that the "labelling of products enables the consumer to make an informed choice and facilitates fairness of transactions between seller and purchaser". Legislative measures may be seconded by certification processes to ensure identity preservation of a given product (seed, food, feed) derived from agricultural commodities. Generally, using the traceability systems established for food and feed, products may be traced back from fork to farm. Depending on the definition of plant varieties developed through novel breeding techniques as GM/non-GM the relevant measures apply, including traceability according to the GMO legislative framework. Alternatively, the variety may be classified as non-GM. To ensure in such cases that the application of a novel breeding technique can be traced back, the applicant has to provide the adequate information during the variety registration process. Traceability is largely based on documentation; detection methods are useful for surveillance purposes.

Traceability according to the European legislation is defined as follows:

- "Traceability means the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution" according to Article 3 (15) of Regulation (EC) No 178/2002.
- "Traceability means the ability to trace GMOs and products produced from GMOs at all stages of their placing on the market through the production and distribution chains" according to Article 3 (3) of Regulation (EC) No 1830/2003.

General traceability requirements are laid down in Regulation (EC) No 178/2002:
According to Article 18 (1), "the traceability of food, feed, food-producing animals, and any other substance intended to be, or expected to be, incorporated into a food or feed shall be established at all stages of production, processing and distribution".
Article 18 (2) of Regulation (EC) No 178/2002 foresees that "food and feed business operators shall be able to identify any person from whom they have

been supplied with a food, a feed, a food-producing animal, or any substance intended to be, or expected to be, incorporated into a food or feed. To this end, such operators shall have in place systems and procedures which allow for this information to be made available to the competent authorities on demand." Finally, according to Article 18 (3) of Regulation (EC) No 178/2002 "food and feed business operators shall have in place systems and procedures to identify the other businesses to which their products have been supplied. This information shall be made available to the competent authorities on demand."

Whereas general traceability systems apply for food and feed, specific legislative measures have been set into force concerning the traceability of GMOs in food and feed. Directive 2001/18/EC foresees traceability and labelling of GMOs "as or in products" at all stages of placing on the market as well as measures to ensure traceability which lie in the responsibility of the Member States. According to this Directive, the notifier has to propose appropriate labelling of the GM product. Since Regulation (EC) No 1829/2003 and Regulation (EC) No 1830/2003 were set into force, detailed rules for labelling and traceability of GM food and feed apply.

The detailed definitions and provisions concerning "the traceability of traceability of food, feed, food-producing animals, and any other substance intended to be, or expected to be, incorporated into a food or feed" (Article 18 (1), Regulation (EC) No 178/2002), "traceability at all stages of the placing on the market of GMOs as or in products authorised under part C of this Directive" (Recital 42, Directive 2001/18/EC), "traceability and labelling of genetically modified organisms and traceability of food and feed products produced from genetically modified organisms" (Recital 23, Regulation (EC) No 1829/2003).

The aim of these legislative measures is to ensure "that relevant information concerning any genetic modification is available at each stage of the placing on the market of GMOs and food and feed produced therefrom and should thereby facilitate accurate labelling" according to recital 23 of Regulation (EC) No 1829/2003. Following Regulation (EC) No 1830/2003, Commission Regulation (EC) No 65/2004 was set into force, implementing a unique identifier for GMOs that are "authorised for the placing on the market in accordance with Directive 2001/18/EC or other Community legislation, and applications for placing on the market under such legislation" (according to Article 1 (1), Commission Regulation (EC) No 65/2004). Sample checks and testing (qualitative and quantitative) are foreseen in Article 9 of Regulation (EC) No 1830/2003. To allow for control measures, however, "all available sequencing information and reference material for GMOs authorised to be put into circulation in the Community must be available". The competent authorities shall have access to the register, which shall also contain relevant information concerning GMOs that are not authorised in the EU, where available.

The legal provisions ensure that any GM commodity entering the food and feed supply chain is traceable until the end product, given that the relevant information and documentation is provided by the operators. For products derived from using novel techniques and intended for consumption as food and feed, these measures can only apply provided that they fall under the current GMO legislation.

Using the traceability systems established for food and feed, products may be traced back to the seed used for their production, and from the seed to a registered variety. Consequently, also a variety developed through novel techniques could be traced back by documentation, provided that the information on the alteration is provided during the variety registration process. The traceability through documentation is ensured starting from the variety registration process. Following the current legislative measures, the applicant for variety registration has to state whether the variety is GM or not; this information also has to be provided during the seed certification process. Product certification procedures (like identity preservation, IP) as well as appropriate labelling measures may in addition be envisaged.

For the detection of any GMO a method validated by the European Union Reference Laboratory (EU-RL) and certified reference material has to be available. This is usually the case if an applicant has launched the application procedure for authorisation of a GMO within the EU. If a validated method along with reference material is available, the traceability and labelling of GMOs as foreseen in Regulation (EC) No 1830/2003 is ensured through lab-based quantification methods. If plants developed through novel techniques are classified as GM the current measures need to be evaluated concerning the practical applicability. In case the prerequisites for detection (method and reference material) are not available, traceability can only be achieved through the Member State traceability systems as required according to Regulation (EC) No 178/2002.

The unintended presence of GMOs is also published in frame of the Rapid Alert System for Food and Feed (RASFF). The severity of any RASFF alert is evaluated on a case-by-case basis. For GMOs, the severity mainly depends on the status (authorised/unauthorised) of the identified GMO. Generally, the presence of unauthorised GMOs in food shall be avoided (zero tolerance) (Regulation (EC) No 1830/2003), as well as the presence of unauthorised GMOs in feed that are not subject to Regulation (EC) No 619/2011. Commodities produced in third countries, in which GM plants are cultivated, are imported into the EU in significant quantities for the use in the feed sector. Thus, in case of feed a higher likelihood of the presence of GMOs than in other sectors related to the production of foodstuffs is anticipated. Commission Regulation (EC) No 619/2011 lays down the methods for official control of feed as regards presence

of GM material for which the authorisation has expired or for which an application has been pending for more than three months under Regulation (EC) No 1829/2003, and which is authorised for commercialisation in a third country. The GM material has not been identified by EFSA as susceptible to have adverse effects on health or the environment when present under the MRPL (minimum required performance limit) of 0.1%. An event-specific quantitative method of analysis already validated by the EU-RL must be published. Certified reference material must be available for official control laboratories.

12 Risk assessment of plants produced by diverse methods, with a focus on cisgenic plants

According to Regulation (EC) No 1829/2003 a risk assessment based on scientific evaluation of the highest possible standard is the prerequisite for authorisation of a genetically modified food/feed within the EU. Article 5 (8) of this Regulation lays down that the European Food Safety Authority (EFSA) shall publish detailed guidance to assist the applicant in the preparation and the presentation of the application. To this end, EFSA Guidance Documents (EFSA 2010; EFSA 2011) summarize the minimum data requirements for each part of the risk assessment. Here, the term "risk assessment" is defined as "hazard assessment", whereas exposure assessment is not included as it is out of scope of the present report.

The regulation of transgenic crops is based on specific "events" (specific transgenic insertions into the host genome). Based on the EFSA Guidance Documents, notifiers and applicants have to prepare separate regulatory data packages for each event.

However, current risk assessment practices of transgenic plants and derived food and feed may be based on inadequate and inaccurate information, and thus cannot achieve the high standards. Molecular characterisation of the genetic modification which is a basic requirement for GMO risk assessment frequently makes use of Southern analysis to detect size and copy number of all detectable inserts. Nucleic acid sequencing, however, would make results more reliable. Long term tests in animals and testing of detrimental effects on reproduction and development of the GM food is usually not required either.

Recently published EFSA Guidance Documents (EFSA 2010; EFSA 2011) and the adaptation of the regulatory framework may further improve the risk assessment process forcing applicants to provide more accurate data and to carry out more tests. However, some important issues are still not sufficiently addressed (e.g. unintended effects, long term effects). Against this background, it is favourable to strengthen the risk assessment of GM plants and to provide reliable answers on all important questions of food/feed safety.

It has to be noted that most of the risks associated with transgenic techniques are relevant for intragenic or cisgenic approaches as well. Intragenesis is not

different from transgenesis except that all parts of the inserted DNA are derived from a cross-compatible species. Intramolecular recombination (inversion, excision, deletion) prior to insertion is not limited by definition. Anti-sense orientation is possible. Cisgenesis, in contrast, is defined as the insertion of a whole, unchanged gene of interest (including introns and regulatory elements) derived from a cross-compatible species. The DNA strand must be inserted in sense orientation.

Here, a closer look at potential differences and similarities of transgenesis and cisgenesis with respect to the different steps of the current risk assessment procedures as set out by EFSA (2011) is given. The discussion of risk assessment does not include exposure assessment, as this issue has not been part of the present assignment.

The first step in risk assessment of GM plants is the molecular characterisation. It has to provide sufficient information relating to the genetic modification and to the GM plant. This, of course, is a crucial point for risk assessment of cisgenic plants, as only a solid characterisation of the DNA sequence of the insert and the flanking sequences can actually prove the cisgenic character of a transformed plant. Similarly, the developer of the cisgenic concept mentions that a molecular characterisation confirming that the plant contains only the intended modifications should always be executed (Schouten et al. 2006). It can therefore be concluded that the molecular characterisation has to be at least as substantial as it is for transgenic plants (Table 3).

Table 3. Details on the information required for the molecular characterisation, according to the EFSA Guidance Document (EFSA 2010)

(EFSA 2010)	Rationale
the methods used for the genetic modification	to help assess the likelihood for and characteristics of transformation-induced mutations, to give insight into the mechanisms of transformation
the source and characterisation of nucleic acid used for transformation	to help substantiate the cisgenic character of the inserted nucleic acid
the nature and source of vector(s) used including nucleotide sequences intended for insertion	to characterise the transformation process, to demonstrate the absence of foreign recombinant DNA
the trait(s) and characteristics which have been introduced or modified	to provide general information about the new plant line

(EFSA 2010)	Rationale
information on the sequences actually inserted/deleted or altered	to fully describe and demonstrate the cisgenic character of the GM plant
information on the expression of the inserted/modified sequence	to check for potential overexpression of the cisproteins
genetic stability of the inserted/modified sequence and phenotypic stability of the GM plant	to verify the stable integration of the cisgene cassette

In trans- and cisgenesis, the same genetic modification techniques are used. Mostly, *Agrobacterium*-mediated transformation is the method of choice for production of cisgenic plants (Lusser et al. 2011). There is scientific consensus that this method creates mutations like deletions and rearrangements of plant genomic DNA (Schouten and Jacobsen 2007, Wilson et al. 2006).

As there is no difference to transgenesis regarding the possibility of unintended effects, a comprehensive molecular characterisation of cisgenic plants modified by an established genetic transformation technique like *Agrobacterium*-mediated transformation or particle bombardment is always necessary.

Substantial equivalence between a transformed plant and its conventional comparator is usually seen as proof for the safety of a GM event. Equivalence tests include multiple fields testing for comparison of different phenotypic characteristics like composition and agronomic parameters.
Generally, also a cisgenic plant could differ substantially from its conventional comparator, as a novel gene derived from a cross-compatible species could disturb the plant's metabolism. Furthermore, negative effects are possible that would not become evident under laboratory conditions but have to be studied on large scale, *i.e.* in the field. Comparative tests are an important tool; both, to strengthen the results of molecular characterisation and to confirm the absence of any unanticipated effects caused by the genetic modification process.
It is entirely possible that cisgenic transformation results in plants not substantially different in phenotypic characteristics, and thus posing similar risks for human and animal health as traditionally bred plants. This question can be answered with reliable certainty only if comprehensive comparative analyses between a cisgenic plant and its conventional counterpart – based on state of the art field designs using powerful statistical approaches – are conducted.

The toxicological and allergological risk assessment of GM plants should provide sufficient information to allow for the conclusion whether or not the derived food and feed has the potential to harm humans and animals.
Regarding the risk assessment of newly expressed proteins, there are some aspects that should be considered. Cisgenic plants as per strict definition express proteins that originate from cross-compatible species only. Proponents of cisgenesis argue that therefore cisgenic proteins are safer than proteins expressed in transgenic plants (Schouten et al. 2006). It is noted however that, with respect to newly expressed proteins, the difference may be wider between two transgenic plants than between a transgenic and a cisgenic plant. This is the case if transgenic plants do not express any new proteins as, for example, the genetically modified starch potato Amflora.
Moreover, it is not clear how unambiguous the definition of cisgenesis is in terms of food safety, as it may not exclude wild relatives that are not part of the human diet so far that can only be crossed under laboratory conditions. A number of food safety aspects that are key to novel transgenic varieties, such as the safety assessment of the expressed proteins, may indeed be much less relevant for these cisgenic plant varieties, as the genes involved were already within the available gene pool when using traditional breeding strategies. If the (distant) relative is also being used as a food source, the safety assessment of the newly introduced protein may obviously benefit from the knowledge that it is already part of the human diet. The food safety assessment should take this into account and be conducted accordingly.
On the other hand, the wild relative may not form part of the human diet yet, and in that case it would be prudent to assess the safety of the newly introduced sequences and protein(s). At any rate, it will be necessary to check for overexpression of newly expressed proteins caused by gene-gene interaction and epigenetic effects.
For the same reasons it is necessary to provide sufficient information also on the expected intake of the food/feed derived from cisgenic plants.

The toxicological and allergological risk assessment also requires testing of whole food and/or feed derived from GM plants "if the composition is substantially modified, or if there are any indications for the potential occurrence of unintended effects" (EFSA 2011). Since it is not further elucidated what "substantially modified" actually means, it cannot be excluded that cisgenic transformation results in substantially modified plants, for example GM plants containing large numbers of stacked genes. Multiple gene stacking in connection with cisgenic plants has been mentioned in the scientific literature (Haverkort et al. 2009).
Furthermore, other aspects such as unintended effects in the new plant variety as a result of insertional mutagenesis will not be different for cisgenic varieties compared to new transgenic varieties.

In contrast to natural breeding and mutation breeding, using cisgenic methods means that a trait is forced into a plant circumventing natural recombination processes as, for example, DNA repair mechanisms within the genome. Thus, the organism is not able to adapt to the changes in genome and the gain of time is probably won at the cost of the plant's health.

Even though with regard to a given species' gene pool, cisgenesis is equivalent to traditional breeding, there are differences, as recombinant DNA technology is certainly not the same as meiotic recombination. However, concerning the environmental risk assessment, plant-to-plant gene transfer may be reconsidered as the introduced gene has already been part of the sexually compatible gene pool.
In principle, the environmental risk assessment (ERA) procedure verifies whether the GM plant shows characteristics as intended and that unwanted interactions with the environment are highly unlikely. As generally foreseen for GM plants, environmental risks arising from development and use of plants and derived food and feed developed by the application of new techniques need to be assessed. Except for risks associated with the inserted gene/introduced trait, unintended effects may occur resulting from the transformation techniques (e.g. *Agrobacterium*-mediated transformation), like rearrangements of plant genomic DNA. Such events potentially lead to changes in phenotypic behaviour affecting the environment.

It is also important to note that a much higher risk of adverse effects becomes evident in case a newly developed plant is planned to be cultivated in an area (e.g. European Union, country, region) instead of when plant material is only imported and processed. For such cases, additional points need to be considered as e.g. impacts of the specific cultivation, management and harvesting techniques. Much more attention has also to be paid to any potential for unwanted interactions with target and non-target organisms, gene transfer (horizontal and vertical), and negative effects on soil and biogeochemical processes.

The fact that the genetic insert, the modified gene and, if applicable, the newly expressed protein, stem from cross-compatible species, lowers the chances that potential adverse effects to the environment occur. Due to the fact that the gene has already been present in the cross-compatible population, a number of risks associated with the use of transgenic plants are presumably not relevant.
Additionally, experience with the qualities and attributes of a gene and the protein it codes for in the close relative is available and contributes to the risk assessment.

It is noted, though, that, apart from unintended effects, plants modified by novel breeding techniques (as compared to a conventional crop plant) could still have an increased potential for:

- persistence and invasiveness including plant-to-plant gene flow if the application of the technique (insertion of a cisgene, genetic alterations, etc.) leads to enhanced fitness (e.g. tolerance to herbicides);

- interactions with target organisms if the application of the technique results in plants resistant to pests or pathogens;

- interactions with non-target organisms if the application of the technique directly or indirectly affects populations of non-target species;

- effects on biogeochemical processes if the application of the technique leads to characteristics influencing such processes (in particular if the modified crop plant is planned to be cultivated on a large scale).

In contrast to the above mentioned points, risks in relation to the transfer of genes to micro-organisms seem to be negligible due to the absence of foreign DNA as e.g. antibiotic resistance marker genes or DNA sequences from microbes.

Firstly, the donor sequence is inserted into the genome at an *a priori* unknown position, which might affect DNA methylation and other factors that in turn can influence gene expression.
Secondly, the insertion of a cisgene results in a mutation at the insertion site. Rearrangements or translocations might occur in the flanking regions. These mutations might knock out genes, open new reading frames and thereby induce phenotypic effects.
Additionally, cisgenic plants should still be tested to confirm that they contain only the intended modifications and no foreign genes, such as a backbone gene from a plasmid. If such a foreign gene is unintentionally introduced, the plant is, by definition, transgenic.

In conclusion, it can be said that the current EU regulatory framework for genetically modified food and feed as well as the respective EFSA Guidance Document (EFSA 2011) in general will be applicable also for plants genetically modified by using cisgenic or intragenic techniques.

13 Model procedures, case studies

By definition, cisgenesis comprises the insertion of an unmodified genomic sequence encoding for the gene of interest, including all regulatory elements, in sense orientation. The sequence originates from the same or a cross-compatible species. Also by definition, a cisgenic plant does not contain any foreign sequences, e.g. marker genes, *Agrobacterium*-derived sequences, vector sequences etc.

Consequently it is of utmost importance to prove the cisgenic character of a plant by providing full characterisation of the genetic cassette and its flanking regions.

Furthermore, the risk assessment needs to address the stability of the introduced trait and potential unintended effects (following comparative, toxicological/allergological and environmental assessment).

A detection system can be developed only if the specific modification is known and this information is accessible to the control laboratory. This is the case if a plant is classified as GMO according to Directive 2001/18/EC. In compliance with European legislation, the applicant has to submit a detection method to identify the transformation event unequivocally in plant material and, where applicable, for its identification, detection and quantification in food and/or feed produced from it; for this purpose, also reference material must be available (see Regulation (EC) No 1829/2003). The European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF, formerly named Community Reference Laboratory) is responsible for validating and testing the methods submitted by the applicants for authorisation (Aguilera et al. 2009).

For traceability of a specific modification the classification as GM/non-GMO is necessary. If classified as GM, all EU regulatory measures concerning GMOs apply, and traceability and labelling is regulated. Traceability based on continuous documentation of the GM-status is possible via traceability systems in seed, food and feed, irrespective of the possibility to detect a modification.

Cisgenic apple (Vanblaere et al. 2011), cisgenic barley (Holme et al. 2011) and ODM maize (Zhu 2000) have been chosen as models for evaluations in practice concerning cisgenic plants, and plants modified using ODM. The papers at hand present information of different extent and thus represent relevant examples to highlight potential difficulties that may be experienced in practice.

13.1 Apple

13.1.1 Scab-resistant apple

Vanblaere et al (2011)

Background

The study aimed at the development of scab-resistant lines of the scab susceptible apple cv. Gala by introducing the endogenous apple scab resistance cisgene *HcrVf2*, a receptor-like kinase mediating resistance against the fungus *Venturia inaequalis*. The major advantage of transgenic or cisgenic methods is that the particular characteristics of a given cultivar are maintained. Furthermore, the breeding time is significantly reduced as compared to introgression breeding.
Several endogenous resistance genes are known; however, only *HcrVf2* has been isolated and shown to be functional.

Construct design, transformation and result

The entire ORF of *HcrVf2* (2943 bp) with its 5' UTR (242 bp) and its 3' UTR (220 bp) was inserted into the pMF1 vector. *Agrobacterium* strain EHA105 was used to transform young leaves of four week old in vitro shoots. Selection of transformants was done on kanamycin-containing medium, followed by the regeneration of marker-free shoots employing a dexamethasone (DEX)-inducible recombinase system. Single integration events were proven with Southern blot analysis with an *npt*II probe in the transgenic mother lines, from which cisgenic lines were developed. The successful elimination of the marker genes was verified by negative selection on 5-fluorocytosine (5-FC)-containing medium. Finally, cisgenic shoots were grafted on M9 rootstocks. The absence of backbone elements was tested by PCR analysis.
Following a number of PCR (presence and absence of backbone integration, *trfA*, *npt*III, pmf_bb3/pmf_bb4; T-DNA integration, *HcrVf2*, *codA*) and RT-PCR analyses (*HcrVf2* and *codA* expression) three putatively cisgenic lines were obtained, containing the gene of interest. The cisgenic plants were phenotypically indistinguishable from untransformed apple plants cv. Gala. However, a report on the effectiveness of the introduced resistance is missing.

Risk assessment

In this report, appropriate data is provided to show that one single insertion of the cisgene has occurred in three primary transformants. Sequence data of the insert and the flanking regions are missing and only semi-quantitative data on expression (by RT-PCR) is presented. The analyses either focus on lines before the elimination of the marker genes (PCR, Southern blot) or are later restricted

to PCR-based analysis, only. Therefore, the presented data would have to be supplemented by detailed sequencing data in order to prove that indeed no foreign DNA sequences are present in the putative cisgenic lines. Consequently, the cisgenic nature of the presented lines has not been sufficiently proven in the present report. Also, the insertion site of the cisgene has not been shown, thus not allowing for the assessment whether the integration of the DNA has caused any disruption of endogenous genes. Stable integration of the insert has not been proven.

Detection

Event-specific detection of the insertion is possible if sequence information is provided. In the present report, the construct design is shown and several PCR primers are shown; however, no sequence information is provided. The cisgene was inserted by means of *Agrobacterium*-mediated plant transformation, leading to random integration of the T-DNA. Therefore, the insertion site has to be specified by sequencing of the flanking regions whereas sequencing of the insert in the selected plant line proves integrity of the insert. Based on the sequence information, an event-specific as well as a construct-specific detection method can be developed. For routine analysis, prior information is indispensable in order to render detection possible.

13.1.1.1 General comments

Risk assessment

No data is available concerning a comparative assessment of cisgenic apple and its conventional counterpart, except phenotypic observation of the shoots in the greenhouse. Information on compositional and agronomic performance of the cisgenic apple line should be provided. However, it has to be taken into account that the lifespan of trees is considerably longer than of annual and biennial plants, leading to potential adaptations in the field trial design, for instance.

Currently there are no data on the consequences of cisgenic modifications on the composition of products. Thus, taking into consideration current knowledge, food safety aspects have to be assessed before placing apples produced from the cisgenically modified trees on the market.

The toxicological safety mainly depends on the fact that the HcrVf2 protein has been part of the human and/or animal diet.

Environmental risk assessment would require a thorough analysis concerning persistence, taking into account the long lifespan of apples and the potential interaction with the environment through gene transfer and interactions with target and non-target organisms, including animals that feed on the fruits. Also,

an exceptional long lasting effect of the cisgenic modification in the apple plant can be anticipated.

Detection

Without information, genome-screening may give concrete evidence of the DNA-modification, but this approach is time and cost-consuming. Protein-based detection methods are hampered by the instability of protein expression, which varies depending on many factors like environmental circumstances, cultivation year, etc. Secondly, the origin of the proteins detected – whether from endogenous genes or inserted cisgenes – may potentially not be differentiated, as the sequences might be highly similar.

Traceability and labelling

Provided that the cisgenic apple is classified as GMO, all regulative measures regarding GMOs apply. This includes the availability of a method for unequivocal detection as well as reference material. In addition, labelling rules as defined in the relevant legislation apply. Due to the possibility to identify cisgenic plants by event-specific PCR methods, quantification is possible and labelling and traceability will be following the current legislative provisions.
The GMO-status of a plant (here: cisgenic apple, no decision concerning GMO/non-GMO) includes that the applicant for variety registration has to state that the apple is GM in a crop-specific technical questionnaire. Based on this, it is possible to trace back by documentation a cisgenically modified apple variety through the planting material until the product.

Fire blight-resistant apple

To date there is no peer-reviewed publication describing the successful development of a cisgenic fire blight resistant cultivar. Fire blight, a destructive disease, is caused by the bacterium *Erwinia amylovora*. The disease is currently hard to control and causes major economic losses. Several genes have been identified that are likely involved in fire blight resistance in diverse apple cultivars (e.g. Khan et al. 2006; Parravicini et al. 2011). The genes may be used to produce fire blight resistant cisgenic apple trees as announced by several researchers.

13.2 Barley

13.2.1 Barley with improved phytase activity

Holme et al. (2011)

Background

The authors aimed at improving a particular quality trait by the insertion of extra gene copies from the species itself. By this, the activity of the enzyme phytase should be raised. Phytase increases the content of bioavailable phosphate and is particularly important in feeding stuff; monogastric animals like pigs and poultry have no phytase activity in their digestive tract, and the phytase level of the mature barley grain is inadequate. The breeding for higher phytase activity by classical means is difficult, because the natural allelic variation for phytase activity in known barley cultivars and wild barley is limited. A barley gene (*HvPAPhy_a*) encoding for phytase activity was used to increase enzymatic activity in the grain. The authors reported that the insertion of one additional copy of the endogenous *HvPAPhy_a* gene increased the activity of the respective enzyme up to 2.8-fold in a homozygous cisgenic plant.

Construct design, transformation and result

The coding sequence of the *HvPAPhy_a* is 2266 bp, consists of five exons. In addition, around 2000 bp of the flanking promoter region and 800 bp of the terminator region were included. The authors chose to clone the gene with the terminator oriented towards the left border, in order to avoid that smaller deletions at the left border region affect the regulatory properties of the terminator region.

Immature embryos were transformed with the *Agrobacterium* strain AGL0 using two vectors containing the *PAPhy_a* gene and a hygromycin resistance gene, respectively. PCR analyses showed that successful co-transformation was achieved in 73.6% of the plants; however, intact integration of the *PAPhy_a* T-DNA was observed in less than 40% of the transformants.

Progeny of “cisgenic” plant line Paphy07 resulting from a 3:1 segregation was analysed in more detail, as it contained the intact insert, did not harbour vector backbone sequences, and did not contain either selection markers nor antibiotic resistance genes. It was confirmed that a single insert was present in the plant and that no tandem configurations had occurred. Sequencing of the flanking regions revealed that T-DNA border nucleotides and synthetic nucleotides originating from the construct were additionally integrated into the plant genome (Figure 11). Also, deletions of nucleotides were observed at the

insertion site. In the end, only two plants displaying all important features of a "cisgenic" plant were obtained.

Left T-DNA border region
*Not*I-site Left T-DNA border Left T-DNA border
GCGGCCGCAGGAACGTTTACACCACAATATATCCTGCCA. (25 bp). GTTTACACCACAATATATCCTGCCAAGATCTTACGT
PAPhy07:
GCGGCCGCAGGAAC***GTTTACACCACAATAT***TATCGGCCATACACACACTAAACAGTACGTATTGCATTTCAAGGTTAAATTC

Right T-DNA border region
*Apa*I-site Right T-DNA border
GGGCCCTCGAGTCGACGTTCCT***TGACAGGATATATTGGCGGGTAAAC***TAAGTCGCTGTATGTGTTTGTTTGAGATCCTCTA
PAPhy07:
GGGCCCTCGAGTCGACGTTCCT***TGA***ACTTTCCGGATTACTGTGCATGCTCTCTTGTAATTGTCTTCTTGATTTTTACTGATGG

Figure 11. T-DNA-border-nucleotides (violet) in the plant genome

Phytase activity was determined in the seeds. Variation was attributed to diverse factors.

Risk assessment

The integrity of the insert in plant line PAPhy07 was demonstrated by Southern analysis; no sequence information concerning the insert is given. The authors show a detailed molecular characterisation of the insertion site including the flanking regions, which revealed that unforeseen integrations and deletions of nucleotides had occurred during the transformation process. The absence of vector-backbone sequences was demonstrated. With respect to a thorough molecular characterisation, sequence information on the insert should be provided.

Detection

A specific PCR of the right and left border-flanking areas is described, but this system does not include the insertion-construct itself. With this, the modification of the plant genome is proven, but the information does not allow the event-specific detection.

13.2.1.1 General comments

Risk assessment

In the work done by Holme et al. (2011) it was not possible to generate plants in which only the cisgene and its regulatory regions have been integrated without any further alteration. In addition to the cisgenic insert T-DNA border nucleotides, synthetic nucleotides originating from the construct, and deletions of nucleotides were observed. Deletions and additional insertions of nucleotides are a phenomenon frequently observed with *Agrobacterium*-mediated

transformation. Per definition sensu stricto, a plant containing additional elements would not be considered as "cisgenic": "Cisgenic plants […] do not contain any parts of transgenes or inserted foreign sequences." However, "T-DNA borders may remain in the resulting organism after transformation" (EFSA 2012). The definition of cisgenesis should be specified precisely concerning the presence of additional sequences. If additional insertions or deletions are observed due to the transformation process it has to be tested whether they have any effect on gene expression and the characteristics of the plant.

A description of the transformed plants on phenotypic level is virtually missing. It would be important to include a comprehensive comparative analysis in order to show that the transformed plant line differs from its conventional counterpart only in the cisgenic trait.

Unintended effects were not reported but cannot be excluded due to the random insertion of the cisgene into the plant genome using currently available transformation methods. Feed safety would have to be assessed before placing the product on the market. Potential negative effects on animals fed with barley with high phytase activity as well as potential negative consequences on the environment need to be evaluated.

Detection

The phytase gene is already present in the plant. Therefore, no large stretches of foreign sequences that could be easily captured by DNA-based methods or newly expressed proteins are present. Therefore, a plant modified by introducing an additional copy of an endogenous gene cannot be easily distinguished from an unmodified plant. The only reliably detectable difference compared to an unmodified plant is to be found in the flanking area sequences adjacent to the insertion site.

For plant line PAPhy07, the nucleotide-sequence of the junction between the T-DNA and the flanking genomic regions is available as the flanking regions of the insert were amplified using a DNA walking kit. Sequencing of the DNA-fragments obtained gives the information which is needed to render detection possible.

Based on the knowledge of sequence-information of the insert and its flanking areas, including T-DNA-borders, a PCR-based-detection can be developed. An event-specific PCR reaction with one primer that anneals to the inserted sequence and another primer that anneals to the flanking DNA reveals the presence of the introduced cisgene (see Figure 12). In order to verify the intactness of the "cisgenic" insert, construct-specific-reactions are feasible.

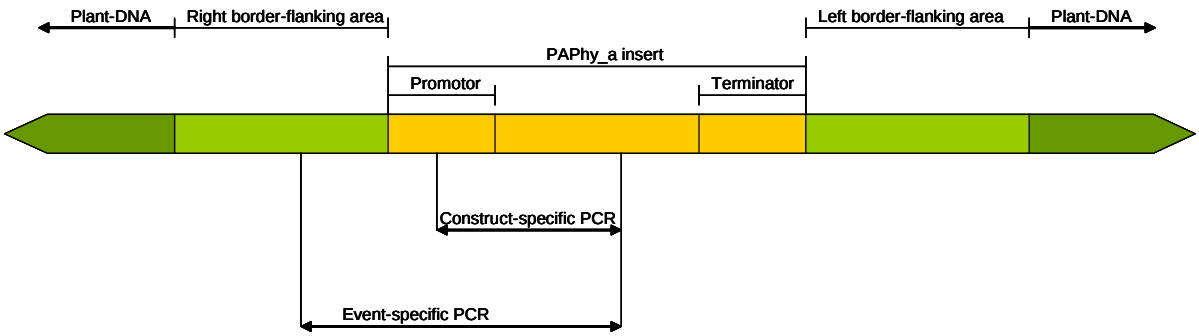


Figure 12. Event- and construct-specific PCR-assay (schematic illustration)

Traceability and labelling

Provided that the plant is classified as GMO, all regulative measures regarding GMOs apply. This includes the availability of a method for unequivocal detection as well as reference material. In addition, labelling rules as defined in the relevant legislation apply. Due to the feasibility to identify cisgenic plants by event-specific PCR methods, quantification is possible and labelling and traceability will be following the current legislative provisions.

If barley harbouring a cisgene is subject to the EU regulatory framework regarding GMOs, the GM-status of a plant includes that the applicant for variety registration has to state that the barley variety is GM in a crop-specific technical questionnaire. Also throughout seed production the GM-status of the barley variety is well documented, including the adequate labelling of the final product. Later on, the systems foreseen for the traceability of food and feed apply. Based on this, it is possible to trace back by documentation a cisgenic barley variety from its registration through seed production to the end-product.

13.3 Maize

13.3.1 Herbicide-tolerant maize

Zhu et al. (2000)

Background

Maize has two families of AHAS genes, AHAS108 and AHAS109, contained in several copies. Several single nucleotide polymorphism (SNPs) have been detected in the maize genome close to the target region; however, no polymorphism was observed in the exact target region. The authors engineered site specific mutations in maize targeting the two sites of the genes encoding for acetohydrocyacid synthase (AHAS108, AHAS109) using chimeric RNA/DNA oligonucleotides targeting both AHAS108 and AHAS109 simultaneously.

Two chimeric oligonucleotides were designed, which are able to make conversions – C to A and G to T - at well-defined positions. These point mutations led to the appearance of chlorsulfuron-resistant phenotypes. On the molecular level, it is not possible to distinguish between varieties developed through “conventional” mutagenesis and those derived from the application of ODM.

Construct design, transformation and result

Copy numbers of endogenous AHAS genes were determined by Southern blot analysis. Sequences spanning the target sites were amplified and digested and unrestricted fragments were sequenced to verify the desired mutations. Chimeric RNA/DNA oligonucleotides were designed and synthesized containing both DNA/DNA and RNA/DNA duplex regions with homology to a target locus; thus, mutations may be specifically induced or corrected, based on mismatch repair. A single amino acid (Ser-621-Asn in both AHAS108 and AHAS109) was altered by introducing the synthetic oligo. Friable embryogenic calli were transformed via biolistic bombardment. Plants with single amino acid substitutions were selected on imazethapyr (Pursuit)-containing medium. The progeny was again analysed for herbicide resistance by spraying with Lightning herbicide. Three resistant plant lines were obtained that contained the new trait.

Risk assessment

When employing ODM, it is not expected that foreign gene or other vector sequences are integrated. ODM is foreseen to work in a highly precise, site-specific way, leading to the introduction of a desired modification in the plant genome without any side effects. However, based on the available data it is not possible to conclude on efficiency and specificity of the method. Unintended mutations of DNA sequences similar to the target sequence should be clarified.
The authors described the transformation vector system and the transformation procedure incl. the selection of resistant plants. They also give detailed information on the sequencing of the target site.

Detection

The chimeric oligonucleotide led to a change of serine to asparagine at amino acid position 621 caused by a substitution of a single nucleotide. Single point mutations can be detected by sequencing and other DNA-based techniques, e.g. restriction fragment length polymorphism (RFLP), high resolution melting (HRM) and amplified fragment length polymorphism (AFLP). The focus of the detection method is on the induced mutation itself.

In the present study the gene was associated with loss of a *Bfa*I site; thus RFLP can be employed to check for the desired variation in the gene. The method was also used by the authors to pre-select for the potential mutant allele. Sequencing of the fragment containing the expected modification is a straight-forward method. The point mutation is visualized as a different peak in the sequencing chromatogram that is absent in the wild type.

13.3.1.1 General comments

Risk assessment

The data given by Zhu et al. (2000) does not allow for the assessment of the efficiency and, moreover, specificity of the method, as only a small part of the plant genome was sequenced. Thus, potential unintended mutations cannot be excluded. Similar side effects may also be expected when applying other methods of mutagenesis. Generally, less damage to the plant genome might be expected in case of targeted mutagenesis but confirming data is largely missing. As a modified protein is produced, the safety of this protein should be assessed based on current knowledge.
The focus of the environmental risk assessment should be on the trait, in this case herbicide tolerance. Also, potential persistence of the trait in weed populations caused by plant to plant gene transfer should be taken into consideration.

Detection

In order to check for the point mutation, both the mutation *per se* and its position have to be known. Fragments including the target site of the AHAS gene can be amplified by PCR. Direct sequencing of PCR fragments confirms that the mutations have occurred successfully and reveals a small new peak at the targeted codon on a sequence chromatogram in comparison to PCR products from the wild type. It is not possible to tell where a mutation originated from (e.g. classical mutagenesis, ODM or ZFN). By sequencing entire genomes and comparative analysis of sequences it would be possible to reveal mutations within a genome of interest; however this method is not feasible in routine analysis.

The enzyme activity itself could be measured, as the herbicide would lead to inhibition of the unmodified enzyme (see Kochevenko and Willmitzer 2003). However, the precise mutation cannot be deduced from this information.

Traceability and labelling

If ODM is classified as GMO, all legislative measures have to be fulfilled, including traceability and labelling, and the applicant has to submit a detection method. However, it is not possible to identify the origin of the mutation by molecular biological techniques. Consequently, it is also not possible to determine the source or technique used to obtain the genomic modification without additional information, focusing on the breeding process. Full documentation on the underlying technique is the major prerequisite to trace back any organism resulting from the application of ODM. For commercialized plants, the breeder would have to indicate at the application for variety registration that the variety candidate has been developed by using ODM. This documentation may then be pursued until the end-product, and the respective product be labelled accordingly.

Cisgenic maize

To date, no cisgenic approach for maize has been published. Being a cereal, maize may be regarded similar to barley concerning traceability and labelling requirements during seed production. Also the risk of admixture of cisgenic GM maize in the food and feed supply chains is higher to that of cisgenic GM barley due to its outcrossing potential.

Zinc finger nuclease (ZFN) crops

No case study for ZFN is presented as publications concerning the application of the technology are currently restricted to the laboratory scale. In principle, using ZFN-1 technique, the same considerations as mentioned under ODM maize will apply.

14 Application of new techniques in plant breeding, different regulatory requirements, risk assessment and traceability

14.1 Potential scenarios of regulatory requirements and their consequences

In this chapter two different scenarios are discussed: A) plants produced through new techniques are subject to the current regulatory requirements for GM plants or B) they are not covered by current regulatory requirements for GM plants.
The EU regulatory framework on seed, food and feed universally applies to all commodities and products, independent of the classification of plants produced through new techniques as GM/non-GM. In particular, traceability based on documentation is governed by the relevant legislative measures. In addition, the GMO regulatory framework applies to cisgenic, ODM and ZFN plants within scenario A.

14.2 Cisgenesis

“Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable – sexually compatible – organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation” (EFSA 2012). In contrast to traditional breeding the cisgene is randomly inserted using standard plant transformation methods. Therefore a cisgenic plant can be distinguished from a traditionally bred one, due to the unique combination of the inserted and the flanking sequences in the plant genome.

Scenario A: Cisgenic plants are covered by current regulatory requirements for GM plants; the EU GMO regulatory framework applies.

After authorisation of an event varieties harbouring the same can be registered in the Common Catalogues of varieties. As part of the authorisation procedure a thorough risk assessment following established methodology and procedures is

mandatory. If remaining in the current GMO regulatory framework, cisgenic plants follow the requirements for the risk assessment of transgenic plants.

Most importantly the cisgenic character of the plant under investigation has to be proven. A detailed molecular characterisation of the plant (insert and flanking sequences, genetic stability and expression analysis) is thus indispensable. In particular, the absence of foreign DNA like trans-/intragenic elements or marker genes that may have been introduced into the plant genome intentionally or unintentionally during the transformation process has to be proven. Provided that the plant harbours only the cisgene and is indeed cisgenic according to the definition, the data requirements for risk assessment may be reduced. Case-by-case different parts of the risk assessment may be implemented, enabling simplification of the risk assessment process. Clearly, this approach would lead to reduced costs for the authorisation procedure.

To decide about a feasible reduction of the data package to be provided potential detrimental effects of the cisgenic modification have to be precluded to the best of knowledge. In frame of the underlying evaluation, consumption and factors posing environmental risks have to be taken into account. In this context, the molecular characterisation serves as a useful basis to decide about further analyses. Food safety and environmental aspects in relation to the newly expressed protein(s) have to be thoroughly considered. Allergological and toxicological studies are likely not necessary if the protein has previously been part of the human diet. Given that the gene has been present in the cross-compatible gene pool in a defined environment the environmental risk assessment could be reduced, for instance concerning plant-to-plant gene transfer.

A cisgenic alteration is not captured by standard screening methods. Provided that sequence information is available, a validated method (event-specific Real Time PCR) can be developed to enable detection and quantification. To this end, also certified reference material must be available. If cisgenic plants are classified as GMO, detection is ensured. According to the relevant legislation the applicant has to provide the authorities with a suitable method and reference material. Consequently, following validation and certification, unequivocal detection and quantification of the cisgenic plant in the production chains is possible.

To date, *Agrobacterium*-mediated transformation is the method of choice to produce cisgenic plants, leading to random integration of the cisgene in the plant genome. The development of an event-specific method is possible for all cisgenic plants developed by this method. The modification may be detected based on its integration site and the unique combination of plant genomic and

cisgene-specific sequences. If necessary, quantification of the presence of an authorised GMO is possible by such an event-specific method.

If the insertion does not occur randomly – as would be the case upon combination of cisgenesis and a targeted DNA insertion technique (e.g. ZFN type-3) – the modification would be detectable only if the sequences of the endogenous and the inserted gene are sufficiently different.

In addition to the GMO legislative measures, the general regulatory framework concerning seed, food, and feed applies. Traceability of commodities and products is generally ensured by documentation as foreseen, starting from the variety registration. In addition, a product produced from GMOs has to be clearly labelled: “This product contains genetically modified organisms’ or ‘This product contains genetically modified [name of organism(s)]” (Regulation (EC) No 1829/2003).

Scenario B: Cisgenic plants are not covered by current regulatory requirements for GM plants.

No authorisation procedure is foreseen. The risk assessment is reduced to common breeding standards.

Detection of cisgenic plants is not possible since no prior information on the modification has to be submitted.

Traceability is restricted to the general provisions concerning the traceability of seed, food, and feed. Similar to feed additives in which the use of GMOs cannot be detected in the product, traceability of cisgenic plants can only be based on documentation and certificates. In this case, information on the cisgenic modification has to be given during the application for variety registration, and continuous labelling or at least the appropriate indication in accompanying papers must be foreseen.

14.3 Oligonucleotide-directed mutagenesis (ODM)

ODM leads to targeted point mutations in the genome that affect gene expression or alter the properties of a protein. Similar alterations in the genome may occur spontaneously or be the result of mutagenesis induced by chemicals or by radiation. It is not expected that plants modified through ODM are distinguishable from those obtained through traditional techniques.

Scenario A: ODM plants are covered by current regulatory requirements for GM plants; the EU GMO regulatory framework applies.

If remaining in the current GMO regulatory framework, plants developed through ODM follow the requirements for the risk assessment of GM plants within the authorisation process. Sequence information is available and the characteristics of the mutated plant are thoroughly documented. Off-target effects due to insufficient specificity of the oligonucleotide may occur and have to be considered as part of the detailed phenotypic and molecular analyses.

The ODM technique aims at the modification of endogenous genes; no foreign elements are foreseen to be stably introduced. The original gene pool remains unaffected. The data requirements for risk assessment might be specified case-by-case and, if applicable, reduced, leading to reduced costs for the authorisation process. It may be envisaged to adapt the risk assessment of plants derived from ODM with respect to the specific investigated mutation and the properties of the conferred trait(s). The definition of the risk assessment data requirements could be governed by the specific trait and its characteristics. Food safety aspects have to be evaluated, in particular if the expression of proteins is increased due to the modification. The characteristics of the modified protein have to be considered and are also important for evaluating potential environmental risks.

It is possible to develop a detection method capturing the ODM-induced mutation if information on the target sequence and reference material are available. During the authorisation procedure the applicant has to provide reference material along with a suitable detection method. On the other hand it is not feasible in the majority of the cases to develop a quantification method based on the Real Time PCR technique. Moreover, a single base substitution/alteration may sometimes even hamper detections or minimise the efficiency of the PCR system employed to a non-predictable extent. Thus, the development of a suitable and specific protocol for quantification can be difficult or unfeasible. As a consequence, the observation of thresholds concerning the adventitious or technically unavoidable presence of GMOs can

be seriously impeded. In addition the detected mutation may not be attributed to ODM unequivocally.

Scenario B: ODM plants are not covered by current regulatory requirements for GM plants.

No authorisation procedure is foreseen. The risk assessment is reduced to common breeding standards.

No information on the mutated site is available and therefore the detection of an ODM-induced genomic modification is not possible.

Traceability is restricted to the general provisions concerning the traceability of seed, food and feed. As described for cisgenic plants, the use of ODM during variety development can be traced back by accompanying documentation provided that information on the modification is delivered within the variety registration process. For this, continuous labelling starting from variety registration and seed production until the final product has to be implemented in the context of the general traceability system. General labelling as ODM based on documentation is possible.

Similar to feed additives in which the use of GMOs in the production process cannot be detected in the product, traceability of plants developed through ODM can be based on documentation. In this case, information on the modification has to be given during the application for variety registration, and continuous labelling, at least on accompanying papers, must be foreseen.

14.4 Zinc-finger nucleases (ZFN)

Three types of ZFN have been reported. Whereas ZFN-1 and ZFN-2 lead to minor changes in the plant genome, ZFN-3 is designed to lead to the targeted insertion of DNA stretches of several kbp in length.

Mutations may occur spontaneously, or be a result of the exposure of plants to mutagens. The origin of the modification, i.e. whether resulting from nature or induced, and in particular the method of mutagenesis, may potentially be unidentifiable, particularly regarding modifications induced by ZFN-1 or ZFN-2. For ZFN-3 it depends on both the insertion site and the inserted gene whether such a differentiation is possible or not.

Scenario A: ZFN plants are covered by current regulatory requirements for GM plants; the EU GMO regulatory framework applies.

The authorisation procedure foreseen for GM plants applies to ZFN plants. The molecular characterisation provides the basis for the development of a detection method (which has to be submitted by the applicant), and serves as important information concerning the further elements necessary in the risk assessment process.

As ZFN techniques are used to achieve a wide range of genomic modifications, the expressed traits and their potential effects on human or animal health as well as on the environment are also expected to be diverse. Case-by-case evaluation concerning the necessary elements of risk assessment is applicable to a high degree. For instance, the targeted insertion of sequences avoids issues raised with currently applied standard transformation methods, especially concerning random insertion of sequences that may lead to the disruption of endogenous genes. In some cases, the assessment of specific risk assessment elements might be advisable. The methodology as for transgenic plants serves as the starting point, and potential side effects resulting from insufficient specificity of the ZFN technique have to be excluded.

Adaptations of the risk assessment procedure with respect to the specific trait may be envisaged, primarily concerning food safety aspects and the environmental risk assessment. For ZFN-1 and ZFN-2, extensive data concerning the effect of the mutation on the (expected) expression and properties of the respective protein will be important to decide about the necessary data package. Alternatively, the origin of a gene introduced through ZFN-3 has to be considered, i.e. whether it is already part of the compatible gene pool as would be the case for cisgenes and whether it has been part of the human diet.

Generally, the detection of the genetic modification is possible if the corresponding sequence including the genomic alteration is known. A validated method (event-specific Real Time PCR), and certified reference material are available. However, depending on the characteristics of the ZFN plant under investigation it may be challenging to develop a method for unequivocal quantification; quantification is in particular difficult when only a single nucleotide has been altered. As a suitable and unequivocal method may not always be readily available, provisions concerning labelling thresholds can be difficult to observe. Notwithstanding these constraints ZFN plants have to be labelled as GMO according to the EU GMO-regulatory framework.

Scenario B: ZFN plants are not covered by current regulatory requirements for GM plants.

As there is no authorisation procedure, neither the modified target sequences (ZFN-1, ZFN-2) nor the inserted genes (ZFN-3) are known. No validated method is available and no detection is possible.

No authorisation procedure is foreseen. The risk assessment is reduced to common breeding standards.

Traceability of plants modified employing ZFN techniques is seriously hampered if ZFN is exempt from regulatory requirements for transgenic plants. As no detection is possible traceability relies on continuous documentation applying the general regulatory framework on seed, food and feed. Information on the modification technique employed has to be requested in the application documents for variety registration.

15 Safety assessment of plants developed through cisgenesis: Comparison to the EFSA Scientific opinion

General opinion given by EFSA

The EFSA Panel on Genetically Modified Organisms (GMO) has given a "Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis" (EFSA 2012).

The Panel concludes that, in general, hazards that might result from various plant breeding techniques are related to the sources of genes used, the genes and traits deployed and changes to the genome, and could impact on human and animal health. But, based on the origin of the cisgenes and the fact that the structure (DNA sequence) of the cisgenes in the recipient plant has remained unchanged compared to the donor plant, gene products similar to those in the donor can be expected in cisgenic plants. Therefore, it can be envisaged that, for some elements of the food and feed safety assessment, there may already be sufficient information to complete a particular part of the risk assessment. Thus, in some cases the amount of new data to be generated to complete the risk assessment may be less extensive for cisgenic plants when compared to transgenic products for which background information is often not available. With regard to the exposure assessment of cisgene-encoded proteins and associated metabolites, sufficient information may already exist on the quantities that are safely consumed. For plants derived through cisgenesis, the EFSA GMO Panel considers that the general approach and all elements described in the guidance for risk assessment of food and feed from GM plants (EFSA 2011) is, at the present time, sufficient for the evaluation of cisgenic plants and derived food and feed. However, for the assessment of food and feed products derived from cisgenic plants it can be envisaged that, on a case-by-case basis, lesser amounts of event-specific data are needed. For example relevant information might already be available regarding the nature of the cisgenic traits and/or plant products, experience with the donor and/or recipient plants and the history of safe use and/or consumption.

Comparison to the EFSA position

Comparably to the conclusion presented in our report, EFSA expresses the opinion that, in general, GMOs generated via cisgenesis should be risk assessed just like transgenic plants according to the available guidelines for risk assessment for food and feed purposes or those for transgenic microorganisms.
EFSA concurs that under certain circumstances it might not be necessary to provide the full data package which is required for the risk assessment of transgenic organisms. In our report we came to a similar conclusion.
We also support the case-by-case principle concerning specific requirements for the risk assessment as suggested by EFSA.
We have some reservations against the EFSA position that also genes from the tertiary gene pool (= from naturally not crossable organisms of the same species) should be regarded as cisgenes. From our point of view this position invalidates the basic definition of a cisgene (= gene from a cross-compatible species).
EFSA points out that it is important to check data from molecular characterisation and comparative assessment for unintended effects because the outcomes of the molecular characterisation can provide insight into potential reasons for unintended effects, such as the disruption of endogenous genes resident at the location of the DNA insertion site. We support this position in our report.
We support EFSA's position that there is no need for open reading frame (ORF) searches within the insert as no new internal junctions are generated. However, insert junctions and flanking sequences have to be risk assessed in the same way as is required for transgenes.
EFSA notes that during the safety assessment it has to be considered whether the donor plant has a history of safe consumption and whether it has already been part of the human diet. If a "history of safe consumption as food" is claimed, this should be specifically documented.
Similar to our argumentation, EFSA refers on several occasions to the importance of the difference between cisgenic and intragenic plants
There is consensus between our report and the EFSA that unintended effects on the environment of cisgenic/intragenic plants can be assumed to be similar to transgenic GM plants.
In the same manner as the EFSA opinion, specific toxicity testing may not be required in cases where it is well documented that both the donor plant and the newly expressed proteins in cisgenic/intragenic plants have a corresponding use and history of safe consumption as food and feed, if the intake levels are within a range considered to be safe. However, if the intake levels are outside of this range, further safety assessment is needed.

16 Conclusions

The technical and scientific progress in plant breeding and genetic modification techniques, together with novel construct designs led to new concepts concerning the genetic modification of crops and discussions whether the application of new plant breeding techniques results in plants defined as “genetically modified organism (GMO) according to the relevant EU legislation. The EU regulatory definitions of GMOs foresee the exemption of some genetic modification techniques/methods according to Directive 2001/18/EC “on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed”, i.e. “(1) mutagenesis” and “(2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods”. How fast the new technologies will evolve and how quickly products will make it to the market depend on many factors. Not only the technical and scientific progress but also the European regulatory framework will influence future developments.

Different scenarios are possible: to sustain the current risk assessment practices and leave crops produced through new techniques within the GMO regulatory framework, to adapt the current risk assessment for GMOs, or to exempt the crops under investigation from the GMO regulatory requirements. In any case, food and feed safety aspects have to be generally taken into consideration for all newly developed plants.

In transgenesis, DNA fragments from any organism and in any combination may be inserted into a genome, extending the gene pool of the recipient species and frequently creating completely artificial traits. Cisgenesis and intragenesis are two new concepts proposed for the genetic modification of plants, involving only genes from the plant itself or from a crossable (sexually compatible) species that may be the same species or a close relative. These genes could also be transferred by traditional breeding methods. Importantly, in cisgenic plants the gene of interest will be left contiguous and unmodified, including its introns and all regulatory elements (promoter and terminator regions). In contrast, intragenic plants may contain genetic elements that have been rearranged *in vitro*, allowing also for gene silencing techniques that cannot be attempted in a targeted manner with cisgenic methods. In both concepts additional sequences and foreign genes such as selection marker and vector-backbone genes should be absent or, alternatively, eliminated from either the primary transformants or their progeny.

Several methods generating targeted, site-directed mutations within genomes have been developed. Among these, oligonucleotide-directed mutagenesis (ODM), which is known under various names, has been used extensively. Upon delivery, the oligonucleotides are degraded by the cell within hours, resulting in transient exposure of the cells to the oligo. Whereas the inducing molecules are not heritable they lead to heritable alterations in the genome. A more recently developed technique based on the use of zinc-finger nucleases (ZFN) causes the site-directed mutagenesis of single or few nucleotides in a plant genome (ZFN-1, -2); the ZFN technique may also be designed to allow for the site-directed insertion of longer stretches of DNA (ZFN-3). Both ODM and ZFN target homologous sequences in the genome and are therefore expected to be highly specific. They may lead to the introduction of new genetic information, including point mutations, the reversal of an existing mutation, or deletions; it is also possible to silence genes by these techniques.

Several patents for ODM have been filed, and the technology is commercially available. Plants produced using ODM are expected on the market within the next few years. The list of crops modified by ODM-based techniques is continuously increasing, as companies have started cooperating on traits in a number of crops. Current developments focus on herbicide and, to a lesser extent, on resistance against other pests. In any case it is necessary to have at hand an efficient selection system after the genomic alteration. ZFN-technologies are a relatively new area of research; therefore in the next years some progress is to be expected. However, it is not feasible to anticipate commercial developments in the next few years.

In addition to the widely employed stable transformation of plants by *Agrobacterium*-mediated transformation, *Agrobacterium* may also be used to express genes transiently. Several factors influence the duration of gene expression; however, in all cases the time of expression is limited. Potential applications are molecular farming, which is used to produce valuable proteins in plants, the analysis of gene function, e.g. plant-pathogen interactions, stress tolerance and resistance studies. Generally, integration into the plant genome is not expected, but could happen in rare cases.

The new techniques under investigation can supplement traditional plant breeding techniques. In some cases, only direct gene insertion or targeted mutagenesis offer the possibility to achieve a desired trait in a given plant. This is particularly true for plants with long lifespans like trees, in which resistance breeding may be accelerated by the new techniques. In addition the properties of the products (e.g. taste, appearance) are left unchanged as no additional changes to the genome are expected.

In order to use the novel techniques successfully, the gene(s) encoding for the desired traits have to be fully characterised. Therefore, monogenic traits, e.g. monogenic resistance, are ideal candidates for employing cisgenic methods. However, it has to be taken into account that the combination of multiple resistance genes contributes to the prolonged maintenance of the resistance. Many important traits are the result of genetic interaction. Therefore, gene stacking, either by crossing or by transformation using multigene cassettes is necessary.

In the past, numerous traditionally bred varieties have been developed from plants mutated with radiation or chemicals, or based on spontaneously occurring, selected mutations. As the mutation may also be induced by the use of techniques like ODM or ZFN-1, the origin of the mutation in a given plant may not be clarified. Consequently, information on the technique used to induce a mutation has to be requested within the application procedure for variety registration if this is to be traced back.

All new techniques under investigation may be combined with cisgenic approaches; however, the potential combination is governed by the breeding goal and may be of limited usefulness. ZFN-3 is a promising technique that can potentially be combined with cisgenesis on a large scale (provided sufficient specificity and efficiency). The combination of the two techniques overcomes the random integration of genes associated with current transformation technologies. Consequently, it might potentially hamper the event-specific detectability of the genetic modification, as the newly inserted sequence may not be sufficiently diverse from the replaced endogenous one.

Generally, for all techniques the same transformation methods as in transgenesis are applied, resulting in similar possibilities concerning the occurrence of unintended effects. To overcome transformation and/or regeneration-induced negative side effects, rigorous selection and subsequent breeding programmes similar to traditional strategies are necessary.

Agrobacterium-mediated transformation causes the random insertion of the T-DNA and frequently leads to unintended insertions of additional sequences that are derived from the bacteria themselves, the vector backbone, or result from DNA rearrangements. In addition, deletions may occur. Following the definition of cisgenesis, the accurate elimination of selection marker sequences, if applicable, needs to be assessed. In any case, isolated genes and their regulatory elements are introduced into another position in the plant genome, different from the introgression of genes by traditional methods. Thus, it has to be proven whether the cisgenes retain their anticipated function in an altered genetic background.

Scientific publications indicate that neither the efficiency nor the specificity of the technologies aiming at targeted alterations of plant genomes can be controlled sufficiently. Unintended effects cannot be excluded.

The thorough molecular characterisation is a crucial step in the risk assessment of plants resulting from the application of new techniques, as only a solid characterisation of the DNA sequence of the insert and the flanking sequences can unequivocally prove their anticipated character. The molecular characterisation in principle aims at the verification that indeed the desired modification has occurred in the plant genome and to exclude unintended effects. In addition, it can provide insights into potential reasons for detrimental impacts resulting from the modification, such as the disruption of an endogenous gene at the insertion site or mutations in the genome different from the targeted one. Thus, for all plants produced through new techniques the molecular characterisation has to be as substantial as for transgenic plants. The molecular and phenotypic analyses should complement each other to provide a solid basis for further (case-by-case) decisions concerning the necessary elements in the risk assessment procedure.

The new techniques are largely equivalent to traditional breeding regarding the gene pool. As the gene has already been present in the cross-compatible population, a number of risks associated with the use of transgenic plants (e.g. plant-to-plant gene transfer) are presumably not relevant. Concerning food and feed safety assessment, reduced data requirements may be appropriate if the donor plant has been part of the diet or the characteristics of the modified plant and derived products are not substantially different from what may be expected as a result of traditional methods. If the donor plant (and thus the introduced/mutated gene) has previously been part of the human diet its safe use and consumption may be anticipated. The safety of newly expressed proteins and metabolites, if applicable, has to be established.

The risk assessment of plants derived by new plant breeding techniques should be based on the same principles and requirements (EU regulatory framework, respective EFSA Guidance documents) as applied for transgenic plants. Based on comprehensive considerations the data requirements may be reduced on a case-by-case basis.

Detection of plants developed by novel techniques (e.g. cisgenesis, intragenesis, ODM, ZFN, Agroinfiltration) is virtually impossible without information concerning the site of the genomic alteration. For the detection of any authorised GMO, a method validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) and certified reference material has to be available. This is the case if an applicant has launched the application procedure for authorisation of a GMO within the EU;

lab-based quantification methods as foreseen in the relevant legislation are then available.

The detection of cisgenic modifications is straight forward using standard methods, as an event-specific detection method can be developed, making use of the unique combination of the genomic DNA sequences and the inserted gene; also, the detection of small alterations in the genome is possible. However, it is not possible to identify the origin of the particular change in the DNA, i.e. whether it is the result of conventional mutagenesis or has been introduced intentionally through ODM or ZFN techniques. The development of an unequivocal quantification method can be difficult, depending on the modification.

If no authorisation process according to GMO-legislation is foreseen, the modified plants and derived products (food, feed and seed) could not be detected in the control laboratory, and the only possibility to ensure traceability is by continuous documentation. The application of a new technique could be indicated in frame of the variety registration process, followed by continuous labelling or indication on accompanying papers. Within the established traceability systems, commodities and products may be traced back to the seed used for their production, and from the seed to the registered variety processed with new techniques.

17 Annex

1. Regulatory definitions concerning traceability and labelling

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

(33) That notification should contain a technical dossier of information including a full environmental risk assessment, appropriate safety and emergency response, and, in the case of products, precise instructions and conditions for use, and proposed labelling and packaging.
(42) It is necessary to ensure traceability at all stages of the placing on the market of GMOs as or in products authorised under part C of this Directive.

Article 4
General obligations

6. Member States shall take measures to ensure traceability, in line with the requirements laid down in Annex IV, at all stages of the placing on the market of GMOs authorised under part C.

Article 13
Notification procedure

2. The notification shall contain:
(f) a proposal for labelling which shall comply with the requirements laid down in Annex IV. The labelling shall clearly state that a GMO is present. The words □this product contains genetically modified organisms□ shall appear either on a label or in an accompanying document;

Article 19
Consent

3. The written consent referred to in Articles 15, 17 and 18 shall, in all cases, explicitly specify:
(e) the labelling requirements, in compliance with the requirements laid down in Annex IV. The labelling shall clearly state that a GMO is present. The words .This product contains genetically modified organisms. Shall appear either on a label or in a document accompanying the product or other products containing the GMO(s);

Article 21
Labelling

1. Member States shall take all necessary measures to ensure that at all stages of the placing on the market, the labeling and packaging of GMOs placed on the market as or in products comply with the relevant requirements specified in the written consent referred to in Articles 15(3), 17(5) and (8), 18(2) and 19(3).
2. For products where adventitious or technically unavoidable traces of authorised GMOs cannot be excluded, a minimum threshold may be established below which these products shall not have to be labelled according to the provision in paragraph 1. The threshold levels shall be established according to the product concerned, under the procedure laid down in Article 30(2).

Article 26
Labelling of GMOs referred to in Article 2(4), second subparagraph

1. The GMOs to be made available for operations referred to under Article 2(4), second subparagraph, shall be subject to adequate labelling requirements in accordance with the relevant sections of Annex IV in order to provide for clear information, on a label or in an accompanying document, on the presence of GMOs. To that effect the words “This product contains genetically modified organisms” shall appear either on a label or in an accompanying document.
2. The conditions for the implementation of paragraph 1 shall, without duplicating or creating inconsistencies with existing labelling provisions laid down in existing Community legislation, be determined in accordance with the procedure laid down in Article 30(2). In doing so, account should be taken, as appropriate, of labelling provisions established by Member States in accordance with Community legislation.

Annex IV
Additional information

This Annex describes in general terms the additional information to be provided in the case of notification for placing on the market and information for labelling requirements regarding GMOs as or in product to be placed on the market, and GMO exempted under Article 2(4), second subparagraph. It will be supplemented by guidance notes, as regards i.a. the description of how the product is intended to be used, to be developed in accordance with the procedure laid down in Article 30(2). The labelling of exempted organisms as required by Article 26 shall be met by providing appropriate recommendations for, and restrictions on, use:

A. The following information shall be provided in the notification for placing on the market of GMOs as or in product in addition to that of Annex III:

1. proposed commercial names of the products and names of GMOs contained therein, and any specific identification, name or code used by the notifier to identify the GMO. After the consent any new commercial names should be provided to the competent authority,
2. name and full address of the person established in the Community who is responsible for the placing on the market, whether it be the manufacturer, the importer or the distributor,
3. name and full address of the supplier(s) of control samples,
4. description of how the product and the GMO as or in product are intended to be used. Differences in use or management of the GMO compared to similar non-genetically modified products should be highlighted,
5. description of the geographical area(s) and types of environment where the product is intended to be used within the Community, including, where possible, estimated scale of use in each area,
6. intended categories of users of the product e.g. industry, agriculture and skilled trades, consumer use by public at large,
7. information on the genetic modification for the purposes of placing on one or several registers modifications in organisms, which can be used for the detection and identification of particular GMO products to facilitate post-marketing control and inspection. This information should include where appropriate the lodging of samples of the GMO or its genetic material, with the competent authority and details of nucleotide sequences or other type of information which is necessary to identify the GMO product and its progeny, for example the methodology for detecting and identifying the GMO product, including experimental data demonstrating the specificity of the methodology. Information that cannot be placed, for confidentiality reasons, in the publicly accessible part of the register should be identified,
8. proposed labelling on a label or in an accompanying document. This must include, at least in summarised form, a commercial name of the product, a statement that .This product contains genetically modified organisms., the name of the GMO and the information referred to in point 2, the labelling should indicate how to access the information in the publicly accessible part of the register.

B. The following information shall be provided in the notification, when relevant, in addition to that of point A, in accordance with Article 13 of this Directive:
1. measures to take in case of unintended release or misuse,
2. specific instructions or recommendations for storage and handling,
3. specific instructions for carrying out monitoring and reporting to the notifier and, if required, the competent authority, so that the competent authorities can be effectively informed of any adverse effect. These instructions should be consistent with Annex VII part C,
4. proposed restrictions in the approved use of the GMO, for example where the product may be used and for what purposes,
5. proposed packaging,
6. estimated production in and/or imports to the Community,
7. proposed additional labelling. This may include, at least in summarised form, the information referred to in points A 4, A 5, B 1, B 2, B 3 and B 4.

REGULATION (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed

(23) Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC ([4]) ensures that relevant information concerning any genetic modification is available at each stage of the placing on the market of GMOs and food and feed produced therefrom and should thereby facilitate accurate labelling.
(29) The traceability and labelling of GMOs at all stages of placing on the market, including the possibility of establishing thresholds, is ensured by Directive 2001/18/EC and Regulation (EC) No 1830/2003.

REGULATION (EC) No 1830/2003 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC

(5) The transmission and holding of information that products contain or consist of GMOs, and the unique codes for those GMOs, at each stage of their placing on the market provide the basis for appropriate traceability and labelling for GMOs. The codes may be used to access specific information on GMOs from a register, and to facilitate their identification, detection and monitoring in accordance with Directive 2001/18/EC.

6) The transmission and holding of information that food and feed have been produced from GMOs also provide the basis for the appropriate traceability of products produced from GMOs.

Article 1
Objectives

This Regulation provides a framework for the traceability of products consisting of or containing genetically modified organisms (GMOs), and food and feed produced from GMOs, with the objectives of facilitating accurate labelling, monitoring the effects on the environment and, where appropriate, on health, and the implementation of the appropriate risk management measures including, if necessary, withdrawal of products.

Article 3
Definitions
3. 'Traceability' means the ability to trace GMOs and products produced from GMOs at all stages of their placing on the market through the production and distribution chains,

Article 4
Traceability and labelling requirements for products consisting of or containing GMOs
A. TRACEABILITY
1. At the first stage of the placing on the market of a product consisting of or containing GMOs, including bulk quantities, operators shall ensure that the following information is transmitted in writing to the operator receiving the product:
(a) that it contains or consists of GMOs,
(b) the unique identifier(s) assigned to those GMOs in accordance with Article 8.
2. At all subsequent stages of the placing on the market of products referred to in paragraph 1, operators shall ensure that the information received in accordance with paragraph 1 is transmitted in writing to the operators receiving the products.
3. In the case of products consisting of or containing mixtures of GMOs to be used only and directly as food or feed or for processing, the information referred to in paragraph 1(b) may be replaced by a declaration of use by the operator, accompanied by a list of the unique identifiers for all those GMOs that have been used to constitute the mixture.
4. Without prejudice to Article 6, operators shall have in place systems and standardized procedures to allow the holding of information specified in paragraphs (1), (2) and (3) and the identification, for a period of five years from each transaction, of the operator by whom and the operator to whom the products referred to in paragraph 1 have been made available.

5. Paragraphs 1 to 4 shall be without prejudice to other specific requirements in Community legislation.

B. LABELLING

6. For products consisting of or containing GMOs, operators shall ensure that:

(a) for pre-packaged products consisting of, or containing GMOs, the words 'This product contains genetically modified organisms' or 'This product contains genetically modified [name of organism(s)]' appear on a label,

(b) for non-pre-packaged products offered to the final consumer the words 'This product contains genetically modified organisms' or 'This product contains genetically modified [name of organism(s)]' shall appear on, or in connection with, the display of the product. This paragraph shall be without prejudice to other specific requirements in Community legislation

Article 5

Traceability requirements for products for food and feed produced from GMOs

1. When placing products produced from GMOs on the market, operators shall ensure that the following information is transmitted in writing to the operator receiving the product:

(a) an indication of each of the food ingredients which is produced from GMOs,

(b) an indication of each of the feed materials or additives which is produced from GMOs,

(c) in the case of products for which no list of ingredients exists, an indication that the product is produced from GMOs.

2. Without prejudice to Article 6, operators shall have in place systems and standardized procedures to allow the holding of the information specified in paragraph 1 and the identification, for a period of five years from each transaction, of the operator by whom and to whom the products referred to in paragraph 1 have been made available.

3. Paragraphs 1 and 2 shall be without prejudice to other specific requirements in Community legislation.

4. Paragraphs 1, 2 and 3 shall not apply to traces of GMOs in products for food and feed produced from GMOs in a proportion no higher than the thresholds established for those GMOs in accordance with Articles 12, 24 or 47 of Regulation (EC) No 1829/2003, provided that these traces of GMOs are adventitious or technically unavoidable.

REGULATION (EC) No 178/2002 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 28 January 2002
laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

Article 3
Other definitions
For the purposes of this Regulation:
'traceability' means the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution,

Article 18
Traceability
1. The traceability of food, feed, food-producing animals, and any other substance intended to be, or expected to be, incorporated into a food or feed shall be established at all stages of production, processing and distribution.
2. Food and feed business operators shall be able to identify any person from whom they have been supplied with a food, a feed, a food-producing animal, or any substance intended to be, or expected to be, incorporated into a food or feed. To this end, such operators shall have in place systems and procedures which allow for this information to be made available to the competent authorities on demand.
3. Food and feed business operators shall have in place systems and procedures to identify the other businesses to which their products have been supplied. This information shall be made available to the competent authorities on demand.
4. Food or feed which is placed on the market or is likely to be placed on the market in the Community shall be adequately labelled or identified to facilitate its traceability, through relevant documentation or information in accordance with the relevant requirements of more specific provisions.
5. Provisions for the purpose of applying the requirements of this Article in respect of specific sectors may be adopted in accordance with the procedure laid down in Article 58(2).

2. Example for questionnaire according to Seed Act 1997 (BGBl. I Nr.72/1997, §9)

Contracting:	**Supporting documents, working sheets**	**Proof of identity**
Which seed lots of Basic seed were sown in field production?	Delivery note, Private contract, IT-based information system	Unique lot identity number of basic seed, Unique field identity number
Who was the grower and localisation of field?		
Amount of used sown basic seed lot?		
Size of seed production in hectare?		

Field inspection:	**Supporting documents, working sheets**	**Proof of identity**
What were the results of field inspection?	Field inspection form, IT-based information system	Unique field identity number
Additional necessity before harvest?		

Harvest:	**Supporting documents, working sheets**	**Proof of identity**
Which harvester was used?	Harvest protocol, IT-based information system Cleaning measures according to a standard working procedure	Unique field identity number
Cleaning measures before/after use of harvester documented?		

Transport and take-over of harvested seed at the seed plant:	**Supporting documents, working sheets**	**Proof of identity**
Traceability of unique field identity number?	Transport protocol, IT-based information system Cleaning measures according to a standard working procedure	Unique field identity number
Which means of transport were used (licence plate number)?		
Cleaning measures before/after use of means of transport documented?		
Cleaning measures before/after use of grain hopper documented?		

Seed processing:	**Supporting documents, working sheets**	**Proof of identity**
Cleaning measures before/after use of dryer, aspirateur, trieur, sieve machine, elevator documented?	Seed processing protocols, In-house working lists, IT-based information system Cleaning measures according to a standard working procedure	Unique identity field number, Unique identity lot number, Silo/container number
Traceability of unique lot identity number in silo/container?		
Which silos/containers were used for the storage?		
Cleaning measures after/before use of silos/container documented?		
Quality status of the seed lot (in-house release of seed lot)?		

Labelling:	**Supporting documents, working sheets**	**Proof of identity**
Administration of a label balance sheet	Label balance sheet	Serial number of labels (from … to …), Unique lot identity number

Placing on the market:	**Supporting documents, working sheets**	**Proof of identity**
Does an official notification for the seed lot exist? When was the seed lot marketed? Who was the buyer/customer (name and address)?	Sales documents, Delivery notes, IT-based information system	Unique identity lot number

3. Annex Table 1. Potential cisgenic methods in potato

trait	trait detail	gene	Donator	reference	country	approach
disease resistance	late blight resistance	*RB* (syn. *Rpi-blb1*)	*Solanum bulbocastanum*	Song et.al. 2003, van der Vossen et.al. 2003	USA, NL	overexpression
disease resistance	late blight resistance	*Rpi-blb2*	*Solanum bulbocastanum*	van der Vossen et.al. 2005	NL	
disease resistance	late blight resistance	*Rpi-blb3*	*Solanum bulbocastanum*	Park et.al. 2005	NL	
disease resistance	potato virus x resistance	*Rx1*	*Solanum tuberosum* ssp. *andigena*	Bendahmane et al. 1999	UK	Silencing
disease resistance	potato virus x resistance	*Rx2*	*Solanum tuberosum* ssp. *andigena*	Bendahmane et al. 1999	UK	Silencing
disease resistance	late blight resistance	*R1*	*Solanum demissum*	Ballvora et al. 2002	GER	Silencing
disease resistance	late blight resistance	*R2*	*Solanum demissum*	Jacobson et al. 2009	NL	
disease resistance	late blight resistance	*R3a*	*Solanum demissum*	Huang et al. 2004	NL	
disease resistance	late blight resistance	*R3b*	*Solanum demissum*	Huang et al. 2004	NL	
disease resistance	late blight resistance	*Rpi-blb1*	*Solanum bulbocastanum*	Jacobson et al. 2009	NL	

trait	trait detail	gene	donator	reference	country	approach
disease resistance	late blight resistance	*Rpi-vnt1*	*Solanum venturi*	Jacobson et al. 2009	NL	
disease resistance	late blight resistance	*Rpi-pra1*	*Solanum papita*	Jacobson et al. 2009	NL	
disease resistance	late blight resistance	*Rpi-sto1*	*Solanum stoloferum*	Jacobson et al. 2009	NL	
disease resistance	late blight resistance	*Rpi-abpt*	*Solanum* ssp.	Jacobson et al. 2009	NL	
disease resistance	late blight resistance	*R2-like*	*Solanum* ssp.	Jacobson et al. 2009	NL	
disease resistance	late blight resistance	*R5*	*Solanum demissum*	Huang 2005	NL	
disease resistance	late blight resistance	*R6*	*Solanum demissum*	El-Kharbotly et al. 1996	NL	
disease resistance	late blight resistance	*R7*	*Solanum demissum*	El-Kharbotly et al. 1996	NL	
disease resistance	late blight resistance	*R8*	*Solanum demissum*	Huang 2005	NL	
disease resistance	late blight resistance	*R9*	*Solanum demissum*	Huang 2005	NL	
disease resistance	late blight resistance	*R10*	*Solanum demissum*	Huang 2005	NL, UK	

trait	trait detail	gene	donator	reference	country	approach
disease resistance	late blight resistance	*R11*	*Solanum demissum*	Huang 2005	NL, UK	
disease resistance	late blight resistance	*Rpi-ber1*	*Solanum berthaultii*	Ewing et al. 2000	USA	
disease resistance	late blight resistance	*Rpi-pnt1*	*Solanum pinnatisectum*	Kuhl et al. 2001	USA	
disease resistance	late blight resistance	*Rpi-mcq1*	*Solanum mochiquense*	Smilde et al. 2005	UK	
disease resistance	late blight resistance	*Rpi-phu1*	*Solanum phureja*	Śliwka et al. 2006	POL	
disease tolerance	bruise tolerance	*Ppo*	*Solanum tuberosum*	Rommens et al. 2004	USA	Silencing
pest resistance	nematode resistance	*Gpa2*	*Solanum tuberosum* ssp. *andigena*	van der Vossen et al. 200	NL	
pest resistance	nematode resistance (G. rostochiensis)	*Gro1-4*	*Solanum tuberosum*	Paal et al. 2004	GER	
physiological effect	increased flavonol content	*Chi*	*Solanum tuberosum*	Lukaszewicz et al. 2004	POL	overexpression
physiological effect	increased *β*-carotene content	*Lcy-e*	*Solanum tuberosum*	Diretto et al. 2006	ITA	Silencing
physiological effect	increased zeaxanthin content	*Zep*	*Solanum tuberosum*	Romer et al. 2002	GER	Silencing

trait	trait detail	gene	donator	reference	country	approach
physiological effect	reduced glycemic index	*Sbe I*	*Solanum tuberosum*	Schwall et al. 2000	UK	Silencing
physiological effect	reduced glycemic index	*Sbe II*	*Solanum tuberosum*	Schwall et al. 2000	UK	Silencing
physiological effect	enhanced flavour	*R1*	*Solanum tuberosum*	Rommens et al. 2006	USA	Silencing
physiological effect	enhanced flavour	*PhL*	*Solanum tuberosum*	Rommens et al. 2006	USA	Silencing
physiological effect	reduced heat-induced acrylamide content	*R1*	*Solanum tuberosum*	Rommens et al. 2006	USA	Silencing
physiological effect	reduced heat-induced acrylamide content	*PhL*	*Solanum tuberosum*	Rommens et al. 2006	USA	Silencing
physiological effect	reduced heat-induced acrylamide content	*Asn1*	*Solanum tuberosum*	Rommens et al. 2007	USA	Silencing
physiological effect	reduced heat-induced acrylamide content	*Asn2*	*Solanum tuberosum*	Rommens et al. 2007	USA	Silencing
physiological effect	reduced heat-induced acrylamide content	*Apg1*	*Solanum tuberosum*	Rommens et al. 2007	USA	overexpression

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Neue Konzepte in der Pflanzenzüchtung und der Pflanzentransformation führten zu Diskussionen, ob die aus der Anwendung von neuen Züchtungstechniken hervorgehenden Pflanzen als „genetisch veränderter Organismus“ gemäß den einschlägigen EU-Rechtsvorschriften zu definieren sind. Dieser Bericht geht vom Stand der Technik bei Cisgenetik, Oligonukleotid-gerichteter Mutagenese (ODM), Zink-Finger-Nukleasen (ZFN) und Agroinfiltration und der derzeitigen Gesetzgebung aus. Ziel ist es aufzuzeigen, welche praktischen Konsequenzen die Anwendung der neuen Techniken und ihrer möglicher Kombinationen auf Risikobewertung, Nachweis, Rückverfolgbarkeit und Kennzeichnung in unterschiedlichen rechtlichen Szenarien hat.

New concepts in plant breeding and plant transformation led to discussions whether plants resulting from the application of new plant breeding techniques can be defined as „genetically modified organism“ in accordance with the relevant EU legislation. The present report is based on the state of the art in cisgenesis, oligonucleotide-directed mutagenesis (ODM), zinc-finger nucleases (ZFN) and agroinfiltration and the current legislation. The aim is to demonstrate the practical consequences of the application of new techniques and their possible combinations on risk assessment, detection, traceability and labeling in different legal scenarios.