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New plant breeding techniques

RNA-dependent DNA methylation, Reverse breeding, Grafting

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Summary

The decision concerning the classification of plants produced by new plant breeding techniques and derived products as genetically modified organisms (GMO) or non-GMO results in consequences relevant for the legislation and the market. To date, there is no general consensus concerning definition or interpretation of the status of these plants.

Following the discussion of practical consequences of cisgenesis, intragenesis, zinc-finger nucleases (ZFN) and oligonucleotide-directed mutagenesis (ODM) techniques, and agroinfiltration in a previous study, this study focuses on the discussion of RNA-dependent DNA methylation (RdDM), reverse breeding and grafting (on GM rootstock). Challenges for regulators, and practical consequences resulting from the limitations of the current GMO regulatory framework when applied to plants produced by new plant breeding techniques have been identified.

In RdDM small RNA molecules lead to methylation of specific DNA sequences and thereby alter gene expression. These epigenetic effects may be achieved through stable insertion of a construct or by transient expression. For reverse breeding plants are transformed with a construct for RNA interference (RNAi) which leads to suppression of meiotic recombination. The progeny results from segregation (negative segregants) and therefore does not contain the construct. When grafting non-GM plant tissue onto GM rootstock, the grafted plant benefits from the molecules expressed in the rootstock and transferred to the upper part of the plant. The non-GM part of the plant does not contain the transgene.

It is challenging to assess to which extent potential risks of the plants and derived products produced through new techniques have to be evaluated. For some of the new techniques (RdDM, reverse breeding, ZFN and ODM) the database to draw final conclusions is currently not sufficient. Cisgenesis, intragenesis, agroinfiltration and grafting make use of well-established methods for plant transformation; risk assessment should therefore follow the current guidance documents for GM plants. When the gene has already been within the compatible gene pool and/or has been part of the usual diet of humans and animals, the data requirements for the relevant elements concerning the risk assessment (e.g. plant-to-plant gene transfer, toxicology) may be reduced. Other elements like the transgenerational inheritance of the epigenetic effects induced by RdDM and thus the stability of the trait have to be evaluated on a solid database, which is currently not available. The same applies to the accuracy and efficiency of site-directed mutagenesis methods. For these techniques further research is required. Reverse breeding and RdDM open up questions concerning the status of negative segregants. When the intermediate GM plants have been produced by using standard plant transformation methods unintended effects due to the genetic modification cannot be excluded. These potential effects should be taken into consideration when recommending risk assessment procedures. Finally, the general question of whether the effector molecules produced by the application of new techniques, e.g. siRNAs, pose risks when ingested by humans and animals to date has not been researched sufficiently. The same applies to effects on other organisms (e.g. insects, nematodes) that are exposed to such molecules, including their potential transmission. In conclusion, for all new plant breeding techniques core elements of the current risk assessment requirements for GM plants are mandatory.

In addition to the characterisation of the plants under investigation, the different techniques pose various challenges to regulators, in particular concerning currently existing labelling regimes for GMOs. The commercialised plants and/or their products should, by technical definition, not contain any foreign sequences and thus cannot be identified using standard molecular methods. Consequently, the application of the techniques frequently does not leave unequivocally detectable traces in the final product, or the changes to the genome cannot be distinguished from naturally occurring ones. Moreover, in many cases the resulting

plants are phenotypically similar to traditionally bred ones. In case of grafting the change induced by the transgene harboured in the GM rootstock cannot at all or not reliably be detected in the product. Whenever there is no change to the genome of the plant, detection and traceability on this basis are seriously hampered. Consequently, labelling according to the currently established threshold for food and feed is not possible, and therefore the regulatory framework has to be reconsidered if labelling and thus information on the use of the new techniques is to be ensured.

Zusammenfassung

Die Entscheidung, ob Pflanzen, die durch neue Techniken der Pflanzenzüchtung hergestellt werden, als gentechnisch veränderte Organismen (GVO) oder nicht-GVO eingestuft werden, hat Konsequenzen, die für Gesetzgebung und den Markt relevant sind. Bis heute gibt es keinen allgemeinen Konsens über Definition oder Interpretation des jeweiligen Status dieser Pflanzen.

Nach der Erörterung der praktischen Folgen der Techniken der Cisgenetik, Intragenetik, Zink-Finger-Nukleasen (ZFN), der Oligonukleotid-gerichtete Mutagenese (ODM) und der Agroinfiltration in einer früheren Studie konzentriert sich diese Studie auf die Diskussion von RNA-abhängiger DNA Methylierung (RdDM), Umkehrzüchtung (Reverse breeding) und Veredelung (auf GV-Unterlage). Durch die Produktion von Pflanzen durch neue Techniken der Pflanzenzüchtung ergeben sich Herausforderungen für die Regulierungsbehörden und praktische Konsequenzen durch die unzureichende Abgrenzung innerhalb des aktuellen GVO Rechtsrahmens.

Bei RdDM führen kleine RNA-Moleküle zu einer Methylierung spezifischer DNA-Sequenzen und verändern damit die Genexpression. Diese epigenetischen Effekte können durch stabile Insertion eines Konstrukts oder durch transiente Expression erreicht werden. Bei Reverse breeding werden Pflanzen mit einem RNA-Interferenz-Konstrukt (RNAi), das zur Unterdrückung der meiotischen Rekombination führt, transformiert. Die Nachkommen stammen aus der Segregation (negative Segreganten) und enthalten daher kein Konstrukt. Beim Veredeln von nicht-GV-Pflanzengewebe auf eine GV-Unterlage profitiert die veredelte Pflanze von den Molekülen, die in der Unterlage exprimiert und in den oberen Teil der Pflanze transportiert werden. Der nicht-GV-Teil der Pflanze enthält kein Transgen.

Eine der Herausforderungen ist es zu beurteilen, in welchem Umfang mögliche Risiken der Pflanzen und der daraus hergestellten Produkte bewertet werden müssen. Für einige der neuen Techniken (RdDM, Reverse breeding, ZFN und ODM) ist die Datengrundlage für die endgültige Beurteilung derzeit nicht ausreichend. Cisgenetik, Intragenetik, Agroinfiltration und Veredelung nutzen etablierte Methoden zur Transformation von Pflanzen. Die Risikobewertung sollte daher den aktuellen Leitlinien für GV-Pflanzen folgen. Wenn das Gen bereits im kompatiblen Genpool und/oder Teil der üblichen Ernährung von Menschen und Tieren ist, können die Datenerfordernisse für die relevanten Elemente der Risikobewertung (z.B. Pflanze-zu-Pflanze Gentransfer, Toxikologie) reduziert werden. Andere Elemente wie die generationenübergreifende Vererbung der epigenetischen Effekte, die durch RdDM induziert worden sind und gleichbedeutend mit der Stabilität des Merkmals, müssen anhand einer soliden Datenbasis evaluiert werden; diese ist derzeit nicht verfügbar. Das gleiche gilt für die Genauigkeit und Effizienz der sequenzspezifischen Mutagenese-Verfahren. Für diese Techniken ist weitere Forschung erforderlich. Reverse breeding und RdDM eröffnen Fragen betreffend die Rechtsstellung der negativen Segreganten. Wenn die intermediären GV-Pflanzen durch Verwendung von Standardtransformationsmethoden hergestellt wurden, können unbeabsichtigte Effekte aufgrund der genetischen Veränderung nicht ausgeschlossen werden. Diese möglichen Auswirkungen sollten für eine Empfehlung zur Risikobewertung in Betracht gezogen werden. Schließlich ist die allgemeine Frage, ob die Effektormoleküle (z. B. siRNAs), die durch Anwendung neuer Techniken entstehen, Risiken bergen, wenn sie von Menschen und Tieren aufgenommen werden, noch nicht ausreichend erforscht. Gleiches gilt für die Auswirkungen auf andere Organismen (z.B. Insekten, Nematoden), die solchen Molekülen ausgesetzt sind, einschließlich einer möglichen Übertragung. Zusammenfassend sind für alle neuen Techniken der Pflanzenzüchtung Kernelemente der aktuellen Risikobewertung für GV-Pflanzen obligat.

Neben der Charakterisierung der untersuchten Pflanzen stellen die verschiedenen Techniken die Behörden vor unterschiedliche Herausforderungen, insbesondere in Bezug auf derzeit geltende Regelungen für die

Kennzeichnung von GVOs. Die kommerzialisierten Pflanzen und/oder deren Produkte sollten gemäß der technischen Definition keine fremden Sequenzen enthalten und können daher nicht mit molekularen Standardmethoden identifiziert werden. Folglich hinterlässt die Anwendung der Techniken häufig keine eindeutig nachweisbaren Spuren im Endprodukt oder die Änderung des Genoms kann nicht von natürlich vorkommenden unterschieden werden. Darüber hinaus sind in vielen Fällen die daraus hervorgehenden Pflanzen in vielen Fällen phänotypisch ähnlich den traditionell gezüchteten. Im Falle der Veredelung kann die Veränderung, die durch das Transgen in der GV-Unterlage hervorgerufen wird, nicht oder nicht zuverlässig im Produkt nachgewiesen werden. Immer, wenn es keine Veränderung im Genom der Pflanze gibt, sind Nachweis und Rückverfolgbarkeit auf dieser Grundlage nicht möglich. Folglich ist die Kennzeichnung nach dem derzeit festgelegten Schwellenwert für Lebensmittel und Futtermittel nicht möglich, und daher müssen die rechtlichen Rahmenbedingungen überdacht werden, wenn die Kennzeichnung und damit Informationen über die Nutzung der neuen Techniken gewährleistet werden soll.

1 Background

A number of new plant breeding techniques are under development or already being employed to create plants with novel traits.

The following new techniques of significance have been identified in a joint report of the Institute for Prospective Technological Studies (IPTS) and the Institute for Health and Consumer Protection (IHCP) of the Joint Research Centre (JRC 2011):

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

All of these techniques involve the use of plant biotechnology methods in the process of creating the desired trait. To date, it is being discussed whether all or some of the new techniques give rise to genetically modified plants according to the definition of "genetically modified organism" (GMO) in the EU regulatory framework, in particular Directive 2001/18/EC. The scope of the relevant legislation is currently a matter of debate among regulators. Several reports have been published that extensively discuss issues concerning the classification of plants and products emerging from new breeding techniques (COGEM 2006; COGEM 2009; JRC 2011; NTWG 2012; ZKBS 2012; ACRE 2013). Also there is on-going international discussion on the GM status of those plants (Akademien der Wissenschaften Schweiz 2012; FSANZ 2012; JRC 2012), and inconsistent conclusions have been made by various working groups.

Although their regulatory classification is unclear worldwide (JRC 2012) plant breeders already apply some of the new techniques in their programmes. Correspondent plants are in the late stages of development or are ready to enter the market. One of the most advanced techniques is ODM, which is already commercially advertised, and specific advice on individual products has been published (ACRE 2011; Waltz 2012). For some other techniques, e.g. cisgenesis, commercialisation may be expected shortly. How soon resulting plants will enter the market depends on the scientific and developmental progress but also on regulatory decisions.

Given technical and economic feasibility, considerable potential of the new techniques in agronomy may be anticipated (Akademien der Wissenschaften Schweiz 2012). Currently it is claimed that they will be rapidly adopted as soon as their regulatory status is clarified. The equivalence of the commercialised plants bred by using some of the new techniques and derived products to those emerging from traditional breeding methods will have to be proven.

It has to be taken into consideration that the techniques are partially still subject of intense academic research and thus their potential applications in plant breeding and their adoption will only emerge in the coming years. Depending on the crop and on the desired trait they may have technical and economic advantages compared to traditional breeding methods. However, their technical efficiency and safety issues need to be carefully considered.

Aside from questions of definition of the plants and their products produced by new techniques and their potential classification as GMO would also bring about new challenges for regulators. The EU regulatory

framework related to GMOs clearly defines measures concerning detection, labelling and traceability, and also lays down the obligatory elements of the risk assessment. The specific features of the plants produced by new techniques pose difficulties to fulfil all effective legislative elements governing the use of GMOs in the EU.

In a previous report it was identified that the classification of the techniques, and derived plants and products, is crucial for their identification, detection and labelling (AGES 2012). The conclusions on the necessary elements of the risk assessment procedures are in line with those of the EFSA opinions published to date ("Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis", EFSA 2012a; "Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function", EFSA 2012b).

DNA-based methods allow for the unequivocal identification of a GMO whereas protein-based methods commonly neither allow for the differentiation between different events (Broeders et al. 2012) nor for the identification of a genome modification in case of similar proteins. The latter is to be expected when cisgenes are transferred.

The standard detection method is based on the PCR amplification of a specific fragment or the junction between the transgenic insert and the host genome (event-specific method; Broeders et al. 2012). Generally, the modifications induced by the new techniques cisgenesis, intragenesis, ODM and ZFN may be detected with current methods, provided that the information is available. Presently this is only the case for plants classified as "GMO". For these plants a validated method for detection and quantification must be available (Directive 2001/18/EC, Regulation (EC) No 1829/2003, Regulation (EC) No 1830/2003).

As cisgenesis and intragenesis use the same methods as transgenesis, similar principles of detection like for transgenic plants may be applied. Although the detection of the inserted elements alone is no evidence of the genetic modification, the fact that the construct is randomly inserted provides the possibility to detect the event. Also, a real-time PCR method can be developed. By this, the content of plant tissue containing a specific event in a sample is quantified, which is important for labelling according to the current legislative measures.

Labelling is based on a threshold in food and feed (0.9%, adventitious or technically unavoidable; Regulation (EC) No 1830/2003), and may be hampered if the modification in the genome may not be distinguished from those induced by traditional breeding methods or occurring naturally. This is usually the case for oligo-directed mutagenesis (ODM) or zinc finger nuclease (ZFN) techniques, specifically ZFN-2. Both techniques lead to (point) mutations and do not result in the insertion of DNA at a specific locus. Their detection is possible provided that there is information on the site of the modification.

Traceability remains unaffected when the information on the technique is given at the application for variety registration. At present applicants are not obliged to provide this information. In this context regulatory measures independent of those applicable for GMOs may be developed, e.g. in the Commissions' proposal (COM(2013) 262 final) concerning plant reproductive material and currently under discussion for the Regulation of the European Parliament and of the Council on the production and making available on the market of plant reproductive material (PRM), which is under discussion now, could and should be the basis for anchoring the evaluation/assessment of all novel techniques in plant breeding to ensure a common accepted and unique approach within EU in future by all member states (MS).

The status of plants bred by employing new techniques is still a matter of discussion, not only but particularly in the EU. If these plants will be classified as "GMO" the applicant has to provide full information on the nature of the modification and currently all legislative measures, including risk assessment and labelling, apply.

Despite on-going discussions concerning the nature of cisgenesis, there is consensus that cisgenic plants are similar to transgenic plants, at least concerning the transformation methods (e.g., EFSA 2012). For some aspects of the risk assessment (EFSA 2011a) the same principles as for genetically modified plants (GMPs) apply. In particular, the safety of both intended and unintended changes relies on the same approach and elements as described for GMPs (EFSA 2012). The elements of the environmental risk assessment according to the EFSA guidance (EFSA 2010) are also relevant for and can apply to plants produced by cisgenesis, intragenesis, ZFN and similar techniques (EFSA 2012a; 2012b). Some elements of the risk assessment may be reconsidered in specific cases, e.g. environmental risk assessment of genes that are already within the sexually compatible gene pool ("cisgenes"), or toxicological and allergological risk assessment of gene products that have been part of the usual diet of humans and/or animals.

The new techniques provide plant breeders with the possibility to achieve desired traits in a more targeted way and with fewer alterations to the genome than previously (JKI 2013). Whereas some of the techniques directly modify the base sequence, others act on an epigenetic level, regulating the expression of genes without sequence alterations. The new techniques are potentially more directed and more precise than traditional plant breeding methods. Nevertheless their efficiency and specificity has to be carefully considered. Current data show that the supposedly precise techniques still suffer from technical constraints that may lead to unintended side effects.

1.1 This study

We previously analysed selected new plant breeding techniques (cisgenesis, intragenesis, oligonucleotide-directed mutagenesis, zinc finger nucleases, and agroinfiltration), which are in an advanced level of development and/or ready to enter the market ("Cisgenesis – A report on the practical consequences of the application of novel techniques in plant breeding", AGES 2012). Intragenesis, cisgenesis or the ZFN-3 technique lead to the stable insertion of DNA sequences into the commercialised plants, and thus can be captured by the current legislative framework, whereas the use of ODM, ZFN-1, -2, and finally agroinfiltration (the latter aiming at transient expression) opens up new challenges to regulators.

The general scenarios as identified in the mentioned report are applicable to outline the general consequences resulting from the use of new plant breeding techniques. The question of whether the new techniques should be subject to GMO regulations can be answered through legal and/or scientific evaluation. It has not been the scope of the present study to answer these questions. However, the still debated definition as "GMO" or "non-GMO" in the current context brings about issues concerning the legal context in which the plants under investigation, *i.e*. resulting from the application of new techniques, and derived products should be evaluated. The characteristics of these plants and products may affect the necessary elements of the risk assessment, but also have practical consequences concerning detection and identification, labelling and traceability.

Techniques investigated in the present study

In **RNA-dependent DNA methylation (RdDM)** epigenetic effects are induced by small RNA molecules. Gene expression is altered through methylation of specific DNA sequences. Although the inducing construct may be stably inserted the same effect can be achieved by transient expression. Propagation thus depends on the research setup. In any case the epigenetic effect is inherited for several generations but will eventually disappear.

For **reverse breeding** plants are transformed with a construct for RNA interference (RNAi) that leads to suppression of meiotic recombination. The progeny results from segregation (negative segregants) and therefore does not contain the construct.

The **grafting** technique is based on grafting of non-GM plant tissue onto GM rootstock. The grafted plant benefits from the GM trait expressed only in the rootstock, whereas the non-GM part of the plant does not contain the transgene. (The procedure is also possible *vice versa*, but in this case there is consensus that the resulting plant and its products would be classified as GMO.)

Challenges

Concerning the necessary elements of the risk assessment (e.g. EFSA 2010; EFSA 2011a) a challenge will be to assess to which extent potential risks of the plants and derived products produced through new techniques have to be evaluated. In many cases they should, by definition, not contain any foreign sequences and thus cannot be identified using standard molecular methods. Moreover, in many cases the resulting plants may neither genotypically nor phenotypically be distinguished from traditionally bred ones.

RdDM does not aim at the stable insertion of a construct but the intended effect is to change the methylation pattern of the genome and thereby inducing a desired trait by exploiting epigenetic effects. In contrast to the effects intended in reverse breeding the epigenetic change is inheritable throughout a limited number of generations, resulting in questions concerning the stability of the trait.

Another technique using “transgenic construct driven breeding” (“negative segregants”) is reverse breeding. The inducer line (the intermediate GMP) carries the transgene, e.g. an RNAi construct or a dominant-negative protein, which is segregated out during further breeding (JRC 2012). The final product, *i.e.* the parental lines and the resulting hybrids, should not contain any traces of the GM approach.

Whenever there is no change to the genome of the plant, detection and traceability are seriously hampered. In case of grafting the change induced by the transgene cannot at all or not reliably be detected in the product. In this report we discuss the challenges for regulators, and practical consequences resulting from the limitations of the current GMO regulatory framework when applied to plants produced by new plant breeding techniques.

1.2 Impact of new techniques and developments

1.2.1 European level

The definition of „genetically modified organism, GMO”, together with techniques and methods excluded, may be found in Directive 2001/18/EC. Risk assessment of a genetically modified plant (GMP) is laid down in the EU regulatory framework.

Diverse guidance documents concerning the risk assessment of GMOs have been published by the European Food Safety Authority (EFSA, http://www.efsa.europa.eu). The EFSA guidance documents strictly foresee the amount of information to be provided for plants classified as GMO. For this study the most relevant are “EFSA Guidance Document on the ERA of GM plants” (EFSA 2010), and “Guidance for risk assessment of food and feed from GM plants” (EFSA 2011a). These two have also been evaluated in the EFSA Scientific opinions published to date dealing with the safety assessment of plants developed through new techniques (EFSA 2012a, EFSA 2012b). According to the EFSA guidance documents plants are tested for unintended effects resulting from the transformation process, including the stable insertion of the DNA and its stability over several generations. So far all GMPs commercialised have resulted from the insertion of a transgenic insert containing a regulatory promoter region, a coding sequence (trait), and a terminator (Broeders et al. 2012). This will not be necessarily the case in plants produced by new breeding techniques.

Reverse breeding and RdDM make use of negative segregants. Although not elaborating on the issue, the EFSA Panel on GMO advised that negative segregants should only be used as comparators for risk assessment when a conventional counterpart is not available or may be included additionally to support the risk assessment

(EFSA 2011b). In another statement, EFSA noted that "negative segregants lack a history of safe use and do not allow the assessment of possible unintended effects of the genetic modification" (EFSA 2011c).

Negative segregant (null-segregant)

Plants that are negative segregants lack the transgenic event and can be produced, for example, by self-fertilisation of hemizygous GM plants, or from crosses between hemizygous GM plants and non-GM plants (EFSA 2011c).

The stability of the traits might pose questions related to current regulations (Lusser and Davies 2013). In particular, limited stability concerns RdDM, as although the methylated status (gene silencing) can be inherited over several generations the epigenetic effect will eventually fade out.

The stability of the trait is also one of the elements of the risk assessment according to the relevant EFSA guideline (2011a).

1.2.2 International level

To date, there is no general consensus concerning definition or interpretation as to whether the new techniques and their products should be considered as GMO or not. Their classification as GMO/non-GMO also results in consequences relevant for implementation and the market.

The JRC Institute for Prospective Technological Studies published a report depicting the different regulatory approaches for new plant breeding techniques (JRC 2012). Representative of the European Union and Argentina, Australia, Canada, Japan, the USA, and South Africa were invited to a workshop that was the basis for the report. Currently the status of plants produced by new techniques is unclear among different regulators. The general approaches for the evaluation of organisms through biotechnology vary in the different countries. Similarly, definite regulatory conclusions for the new techniques are largely missing (JRC 2012). In some cases and upon concrete questions the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has issued comments concerning deregulation to companies (e.g. in the case of ODM or ZFN, following a case-by-case approach).

Whereas there was consensus that a GM rootstock is clearly GM the nature of the fruit is still under debate.

Reverse breeding and RdDM make use of negative segregants, *i.e.* the plants themselves do not carry the transgene. The regulatory situation of negative segregants (progeny of a GMO which no longer contains the transgene) is unclear (JRC 2012; Lusser and Davies 2013). The USDA decided on negative segregants resulting from RNAi-transformed plum and sorghum lines on a case-by- case basis (Lusser and Davies 2013). One matter of discussion may be that even though the introduced DNA is not inherited the introduced trait still is. As an example, according to the definition of a GMO in the Australian legislation (Gene Technology Act 2000), the progeny of a GMO – provided that the introduced trait is inherited – is a GMO in this sense (Lusser and Davies 2013). For negative segregants it would also be important to prove the absence of inserted DNA sequences.

2 Literature search – Methodology

For literature search the bibliographic database Scopus, officially named SciVerse Scopus, and the public access database PubMed were used.

Scopus is described as the largest abstract and citation database of peer-reviewed literature, features smart tools to track, analyse and visualize research and delivers a comprehensive overview of global scientific output (online: http://www.elsevier.com/online-tools/scopus; last accessed: 31.10.2013).

PubMed, a database developed and maintained by the National Center for Biotechnology Information (NCBI), provides access to peer-reviewed literature in the fields of biomedicine and health, covering portions of the life sciences, behavioural sciences, chemical sciences, and bioengineering, and additional relevant websites and links to the other NCBI molecular biology resources (online: http://www.ncbi.nlm.nih.gov/pubmed/; last accessed: 31.10.2013).

Literature search started in March 2013 and was finalized in June 2013. Additionally, current and relevant publications were included in retrospect.

Scopus comprises also a patent database SciVerse – Hub, which covers searching in five international databases.

The literature research was performed through search specifically chosen keywords for each method. To improve the searching process Boolean operators and truncation wildcards were used. If no specific information is given, terms were searched in "All fields".

List of keywords and literature results for each method:

RdDM:

Key words:	Hits:
„RNA dependent DNA methyl*" **AND** plant*	22
„RNA directed DNA methyl*" **AND** plant*	1192
RdDM **AND** plant*	141
RdDM **AND** „transgenic plant*"	20
RdDM **AND** TGS	19
„transcriptional gene silencing" **AND** „double stranded RNA" AND methyl* AND plant*	87
„transcriptional gene silencing" **AND** „dsRNA" AND methyl* AND plant*	26
„RNA mediated transcriptional gene silencing" **AND** plant*	7

RdDM (PubMed):

i) Boolean search (exact term)

RNA dependent DNA methyl* **AND** plant*

Title	0	
Title/Abstract	10	(first 600 variations of truncated terms)
All fields	12	(first 600 variations of truncated terms)

RNA dependent DNA methyl* **AND** plants*

Title	0	
Title/Abstract	8	
All fields	9	

RNA directed DNA methyl* **AND** plant*

Title	7	
Title/Abstract	116	(first 600 variations of truncated terms)
All fields	139	(first 600 variations of truncated terms)

RNA directed DNA methyl* **AND** plants*

Title	4	
Title/Abstract	101	
All fields	114	

RdDM **AND** plant*

Title	1	
Title/Abstract	69	(first 600 variations of truncated terms)
All fields	86	(first 600 variations of truncated terms)

RdDM **AND** plants*

Title	0	
Title/Abstract	58	
All fields	69	

ii) Non-Boolean search (exact term)

RNA dependent DNA methylation/RNA-dependent DNA methylation

Title	2
Title/Abstract	15
All fields	15

RNA dependent DNA methylation **AND** plant/RNA-dependent DNA methylation **AND** plant

Title	0
Title/Abstract	5
All fields	14

RNA dependent DNA methylation **AND** plants/RNA-dependent DNA methylation **AND** plants

Title	0
Title/Abstract	8
All fields	14

RNA directed DNA methylation/RNA-directed DNA methylation

Title	57
Title/Abstract	163
All fields	163

RNA directed DNA methylation **AND** plant/RNA-directed DNA methylation **AND** plant

Title	3
Title/Abstract	62
All fields	156

RNA directed DNA methylation **AND** plants/RNA-directed DNA methylation **AND** plants

Title	4
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RNA directed DNA methylation/RNA-directed DNA methylation (*Epigenesis, Genetic* [MeSH])

Title	31
Title/Abstract	99
All fields	99

Reverse breeding:

Key words:	Hits:
„reverse breeding“	24
„crossover control“ AND breeding AND plant*	8

Grafting:

Key words:	Hits:
graft* AND „transg* rootstock*“	23
graft* AND „transform* rootstock*“	15
graft* AND „GM rootstock*“	0
graft* AND „WT scion*“	7
graft* AND „wild type scion*“	18

Documents obtained for each method were screened in order to select in the first step review papers and/or research papers describing basic information of the methods and the use of techniques in plant breeding. Documents were also screened for applications, plants and breeding goals and anticipated developments. Publications with no relevance were not used as references.

2.1 Selection of documents

The elaboration of the characteristics of the different methods investigated in this study is mostly based on selected scientific peer-reviewed literature. The selected key papers may be found, including their abstracts, in the annex. The selection procedures are described below.

RdDM

Publications concerning RdDM were selected according to the significance for evaluating the application of RdDM technique in plants, *i.e.* studies, reviews and experiments related to the application of RdDM in humans and animals, as well as microorganisms were not taken into consideration. Literature concerning post-transcriptional gene silencing and not DNA methylation were also eliminated and reviews separated from experimental papers. Review papers were used for the introduction and basic information. Experimental papers were further screened for "genetically modified plants" and whether tests were performed on success rate and inheritance of the RdDM induced trait.

Reverse Breeding

For this method to date only one experimental paper has been published; no selection procedure applies.

Grafting

For the grafting method, publications were selected according to the determination of the presence or absence of moving transgenic molecules from the GM rootstock to the scion (by passing the graft junction). Lists of research studies in which detection of transgenic molecules was demonstrated and in which detection of transgenic molecules could not be established were created.

3 RNA-dependent DNA methylation

RNAi describes a gene regulation process leading to gene silencing which is dependent on small RNA (Finke et al. 2012; Castel and Martienssen 2013). More precisely, gene silencing is a synonym for two different processes, a cytoplasmatic mechanism called post-transcriptional gene silencing (PTGS), which goes hand in hand with mRNA accumulation, and a mechanism called transcriptional gene silencing (TGS) or RNA-dependent DNA methylation (RdDM), which mainly takes places in the nucleus. Both mechanisms can be used for artificial silencing of target genes.

3.1 Introduction and definition

RNA-dependent DNA methylation (RdDM) is a small interfering RNA (siRNA) based epigenetic modification which induces gene silencing by making promoter regions inaccessible for the transcription initiation complex and represses transposons and retro-elements by the formation of heterochromatin at repetitive loci in the plant genome (Lorkovic et al. 2012; Castel and Martienssen 2013). Active transcription is a requirement for RdDM (= transcriptional gene silencing). Modifications in methylation patterns introduced by RdDM are inheritable and – if occurring in germline cells – passed on to the next generation (Law and Jacobsen 2010).

In general, the methylation of cytosine at position 5 is a common modification of plant genomic DNA that is associated with epigenetic phenomena such as transgene silencing, transposon suppression, maternal/paternal imprinting and paramutation (Finke et al. 2012). RNAi has been proposed to be a natural plant defence mechanism against viruses and transposons and is also involved in the regulation of the expression levels of certain genes (Febres et al. 2008).

Silencing is initiated in plants by dsRNA that is processed into small interfering RNA (siRNA) of about 21-26 nucleotides (nt). Recently, 24-nt small RNAs have been proven to be essential for epigenetic modification, although small RNAs of the other size classes can also have the role. It was shown that in *Arabidopsis* Dicer-like proteins produce 21-nt, 22-nt and 24-nt siRNAs, respectively (Kanazawa et al. 2011), but most of the very large number of identified siRNAs involved in RdDM are 24-nt long (Okano et al. 2008).

In nature, the process can be triggered by a variety of different conditions as stress induction, transposon mobility and, most importantly, viral infection (Dalakouras et al. 2011). However, the silencing process is inhibited by some viruses containing RNAi suppressors as, for example, Citrus tristeza virus, which carries three suppressors of silencing (Febres et al. 2008). In contrast, the RdDM process can also be triggered artificially by transgene expression.

It is so far not clear what factors play a role and what are key elements for maintenance of DNA methylation. Different models have been discussed, and it is proposed that factors as the distribution of the cytosine (symmetrical *vs.* asymmetrical, homogeneous *vs.* heterogeneous, etc.) and also the structure of the transcribed region (e.g. hairpin construct) or secondary DNA structure are highly relevant. The type of plant tissue may also play its role (Dalakouras et al. 2011).

Transgenic gene cassettes which encode dsRNA usually complementary to a specific promoter sequence are transferred by standard procedures into the plant genome (see “Transformation methods” below). These dsRNA molecules induce the methylation of the respective promoter sequence *via* the nuclear RNAi pathway which leads to the silencing of the gene controlled by this promoter. The process implies that at a certain stage during the development of the modified crop it is transgenic.

3.2 Applications

RdDM is applied to induce gene silencing of specific genes in plants without introducing mutational changes in the relevant DNA sequences. The phenotypic alterations are achieved by the methylation of cytosine nucleotides – mainly in GC-rich promoter regions – which leads to the formation of heterochromatin and subsequent inaccessibility of these DNA sequences for RNA polymerases and transcription factors.

3.2.1 Plants, breeding goals and anticipated developments

During traditional plant breeding varieties containing the transgenic RNAi insert can be excluded by segregation to achieve modified plant lines clear of any transgenic DNA but retaining the modified methylation pattern and the gene silencing effect. As examples, the following breeding objectives have been achieved using RdDM:

1. Induction of male sterility in maize by silencing the fertility gene *ms45* (Cigan et al. 2005).

2. Reduction of amylose content in potatoes by silencing the GBSSI gene (Heilersig et al. 2006).

Like the research describes in Cigan et al. (2005) male sterility systems in general may be of interest for species in which breeders would like to establish hybrid systems. In addition, all quality characters and features influencing commercial success of agricultural species and vegetables will gain importance as indicated by the option of creating more pre-harvest sprouting tolerant cereal species (Singh et al. 2013).

RdDM processes have been studied in many different plant species like *Arabidopsis thaliana*, rice, maize, barley, wheat (Singh et al. 2013), tobacco and petunia. RdDM was successfully used for example for the induction of male sterility in maize by inhibiting the expression of the *ms45* fertility gene (Cigan et al. 2005) and for reducing the amylose content of potatoes by silencing the GBSSI gene (Heilersig et al. 2006).

This new methodological approach of gene silencing may enable the realisation of specific requested characters in the cultivars of agricultural species and vegetables. The method might support the development of hybrid production systems in species in which no functioning hybrid system has been established to date. Hybrid systems – as well-established in maize, rapeseed or sunflower – are usually correlated with a significantly higher yield. As these species are important for human and animal nutrition, this possibility for yield increase has an elementary impact on food security.

Especially for species such as barley or wheat, hybrids will become more important in the near future. Due to their nature as self-pollinators with small flowers hybrid production faces some difficulties and commercial production of hybrid seed has just been attempted in a few seldom, using various methods like gametocides.

RdDM is a versatile method opening up infinite possibilities to tailor cultivars for distinct production systems like food, feed, and energy-pathways. In principle any gene can be targeted in any species. The major restriction to use the method for agricultural species and vegetables lies in its efficiency and specificity. As the technique is still under development major impacts on cultivar development may be expected in a medium term.

3.3 Basic principles

RdDM is based on the nuclear RNAi pathway which inhibits gene expression of targeted loci *via* methylation of cytosine and seeding of heterochromatin formation, in contrast to the cytoplasmic RNAi pathway(s) which achieve gene silencing *via* siRNA targeted RNA transcript degradation (= post-transcriptional gene silencing) (Castel and Martienssen 2013; Heinemann et al. 2013).

RdDM was originally described in plants but appears to be evolutionary conserved and is also active in fungi, insects and mammals (Law and Jacobsen 2010). It plays a decisive role in genome stability in somatic cells and during meiosis and gametogenesis in germline cells by keeping transposons silent (Law and Jacobsen 2010; Castel and Martienssen 2013).

The different approaches for RdDM provide a set of tools for targeted methylation of cytosine leading to silencing of particular genes, especially promoter sequences. If the targeted DNA has promoter function, this RdDM can result in transcriptional gene silencing (Finke et al. 2012). Methylation is dependent on the homology between the transcribed sequence (e.g. the virus promoter sequence in case of virus-induced silencing) and the methylated DNA sequence (Febres et al. 2008).

RdDM processes can be induced by transient presence of nucleic acid molecules in cells. Japanese researchers, for example, described the successful introduction of a transient viral vector producing and transmitting siRNA into the nucleus of petunia plant cells. In those experiments, the viral 2b protein was found to be responsible for transmission of siRNA into the nucleus (Kanazawa et al. 2011).

The transient presence of nucleic acid molecules makes RdDM attractive for plant gene technology, since no changes or mutations are introduced into the primary DNA sequence of the modified organism. The desired effect is mediated *via* an epigenetic modification of the expression of the targeted gene. Usually the gene of interest is transcriptionally silenced *via* methylation of the corresponding promoter region(s). In plants the introduced methylation patterns are meiotically stable and inherited for several successive generations. It is, however, not clear for how many generations the effect of gene silencing by RdDM remains in the absence of the inducing construct, as the degree of silencing is not always related to the degree of methylation (JRC 2011).

Another RdDM technology is established using stable gene expression systems as single transgenes, transgenic hairpin structures or inverted repeats that produce promoter specific dsRNA. Although successfully applied in annual as well as in perennial plants (e.g. grapefruit), single transgene systems seem to be the least effective of the three (Febres et al. 2008). Due to breeding and segregation successive generations (negative segregants) will contain the altered methylation patterns without the inserted foreign DNA. However, as mentioned above, over the generations the intended genetic effect may decrease and fade out.

RNA dependent DNA methylation in plants - Modes of action

RNA-dependent DNA methylation in plants was elucidated primarily in the plant model system *Arabidopsis thaliana*. Valuable additional data concerning nuclear RNAi pathways were retrieved from *Schizosaccharomyces pombe*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Castel and Martienssen 2013).

There are obvious parallels between RdDM in *A. thaliana* and co-transcriptional gene silencing in *S. pombe*: For both there is a requirement for active transcription and gene silencing is directed at repetitive heterochromatic genome loci (Castel and Martienssen 2013).

The most prominent difference between both organisms is that *de novo* methylation in plants by RdDM requires a stepwise transcription by two RNA polymerases (RNA Pol IV and V) (Chen 2010). RNA Pol IV transcripts serve as targets for siRNA generation, RNA Pol V transcripts are targeted by the RNAi effector protein complexes itself. The initial templates for RNA Pol IV are still not clearly characterised but they are presumably the targeted repetitive heterochromatic DNA loci subject to RdDM (Onodera et al. 2005; Haag and Pikaard 2011). RNA Pol IV is associated with RNA dependent RNA polymerase 2 (RDR2) which produces strands of RNA complementary to the single stranded RNA Pol IV transcripts (see Fig. 1) (Law et al. 2011). The resulting

double stranded RNA molecules are substrates for the dicer-like 3 complex (DLC3) which cuts the dsRNA into 24-nt siRNA molecules (Kasschau et al. 2007). These siRNAs are exported from the nucleus into the cytoplasm where they are loaded into the Argonaute4 complex (AGO4) which is mediated by heatshock protein 90 (HSP90) (Ye et al. 2012). The loaded AGO4 complex is transferred back into the nucleus where AGO4 targets nascent RNA Pol V transcripts (Zilberman et al. 2003). The interaction is directed *via* sequence complementarity to the processed siRNA elements carried by AGO4. The RdDM complex contains the following components: AGO4, RNA Pol V, the catalytically active *de novo* methyltransferase DRM2, a RNA Pol V associated GW/WG protein KTF1, which is assumed to organize the interaction between AGO4 and 5-methylcytosine in the genomic DNA; and RDM1, which is associated with AGO4, binds to single stranded DNA and interacts with DRM2 (Castel and Martienssen 2013). RDM1 and DRM2 contribute to a positive feedback loop between AGO4 targeting and DNA methylation (Gao et al. 2010). DRM3 is also involved in RdDM; however, its exact function is not clearly defined yet. After association with DRM3, DRM2 appears to be capable of cytosine methylation in all sequence contexts (Castel and Martienssen 2013), *i.e.* symmetrical and asymmetrical cytosine motifs are targeted during RdDM for *de novo* methylation (Pelissier et al. 1999). In contrast to mammalian somatic cells, DNA methylation at asymmetrical cytosine (CAG, CTG, CCG, CAA and CTT) was described in *Arabidopsis thaliana* (Finke et al. 2012).

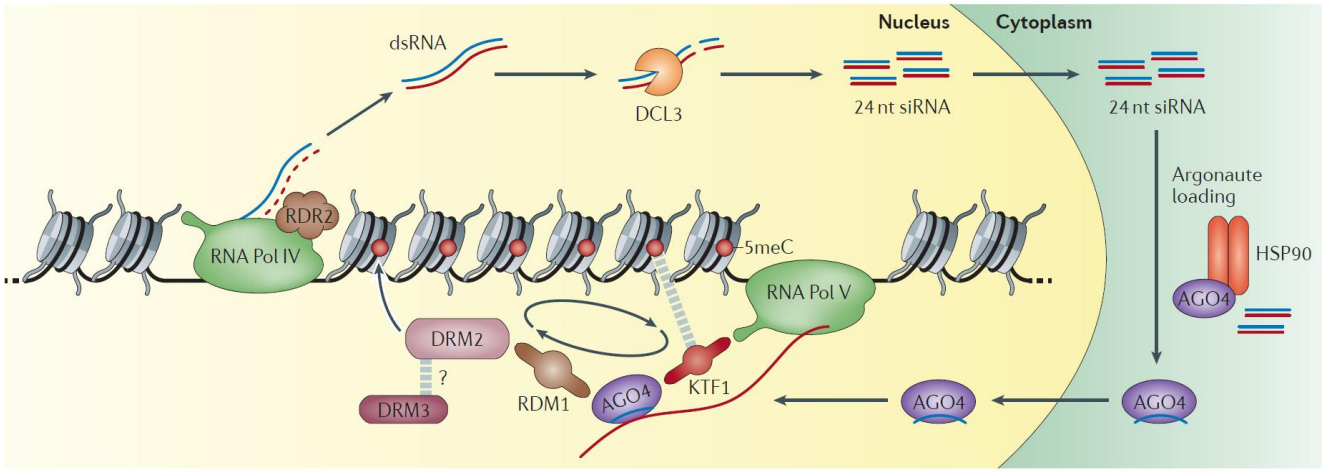

Figure 1: RNA dependent DNA methylation pathway in plants (modified from Castel and Martienssen 2013)

This *de novo* DNA methylation pathway was initially characterised in *Arabidopsis thaliana*. The core steps of the process occur in the nucleus (= nuclear RNAi pathway). Loading of the involved Argonaute RNAi effector proteins proceeds in the cytoplasm. The introduced changes in the cytosine methylation pattern lead to the formation of heterochromatin primarily silencing transposons and repetitive (retro-) elements. The modifications are inherited after cell division if happened in somatic cells or passed on to the next generation if methylation took place in germline cells. The effect is usually stable for several generations but eventually fades out.

RNA Pol IV:	RNA polymerase IV; transcription of target RNA
RDR2:	RNA-dependent RNA polymerase 2; synthesis of RNA strand complementary to the target
dsRNA:	double stranded RNA
DCL3:	Dicer-like 3; production of 24 nt siRNAs
AGO4:	Argonaute 4; targeting of methylation apparatus to genomic loci to be silenced
HSP90:	heat shock protein 90; loading of 24 nt siRNAs to AGO4
RNA Pol V:	RNA polymerase V; de novo transcription of DNA region to be silenced
KTF1:	Kau domain –containing transcription factor 1 (similar to transcription factor SPT5[40]);
RDM1:	RNA-directed DNA methylation 1; binds to methylated ssDNA, fixation of RdDM complex to target region, bridging RNAi and methylation[1]
DRM2:	domains rearranged methyltransferase 2; major *de novo* methyl transferase
DRM3:	domains rearranged methyltransferase 3; non-functional methyl transferase

Some differences in RdDM processes are known to have been successfully observed and described in plants, which reflects the complexity of interactions at molecular level during RdDM in living cells. For example, in

maize transposable elements that were highly methylated seem to be devoid of siRNAs indicating that siRNAs at some loci are not required for induction of the RdDM (Dalakouras et al. 2009).

In one process RNA polymerase IV mediates the production of dsRNA (Castel and Martienssen 2013), but another one involves RNA polymerase II and transgenic hairpin RNA, and leads to the production of 24-nt siRNA *via* 120-150-nt dsRNA intermediates (Dalakouras et al. 2009).

In another RdDM mechanism siRNA is transported in the nucleus by the viral 2b protein without the help of argonaut proteins (Kanazawa et al. 2011). Also, siRNAs are not always homologous with repetitive sequences. On the contrary, many endogenous plant siRNAs have homology with non-repetitive sequences suggesting that repetitive and non-repetitive sequences are associated with endogenous siRNAs (Okano et al. 2008).

RdDM can be applied to induce methylation of promoter regions as well as for coding region methylation. The latter was thought to be meiotically lost in the absence of an RdDM trigger, but more recently it was observed that cytosine methylation of symmetrical motifs (CG, CHG) was maintained in a transgenic region. The maintenance of different cytosine methylation patterns is associated with different enzymes. In *Arabidopsis* methyltransferase 1 (MET1) was found to maintain and *de novo* methylate CG motifs, and "domains rearranged methyltransferase 2" (DRM2) was found to be a major *de novo* DNA methyltransferase (Miki and Shimamoto 2008). In contrast, chromomethylase 3 (CMT3) maintains CHG motifs, with H standing for C, A and T.

Silencing experiments targeting the transgenic 35S-GFP (green fluorescent protein) promoter indicated that methylation of CHH could be maintained in T1 progeny in rice. It is also suggested that there may be maintenance of methylation asymmetrical cytosine independent of siRNA, *i.e.* silencing is epigenetically inherited (Okano et al. 2008).

The methylation of a promoter sequence attracts histone modification marks leading to heterochromatinisation. Chromatin modifications of targeted promoters have been reported in *Arabidopsis* and rice (Miki and Shimamoto 2008). Specifically, DNA methylation in promoters triggers histone deacetylation and methylation which then supports transforming chromatin to transcriptionally inactive heterochromatin and maintenance of methylation patterns (Dalakouras et al. 2011). It has been shown that RdDM is correlated with histone modifications involving histone H3 lysine 9 dimethylation on the target sequence (Kanazawa et al. 2011).

For the risk assessment of plant breeding technologies relying on RdDM it is relevant to realise that there is evidence that RdDM is also capable of acting *in trans* in plants. One of the best known examples is the phenomenon of "paramutation" which was initially described in maize: Silencing of one allele (= the paramutated) is mediated in heterozygous combinations by an already silenced allele (= the paramutagenic) *in trans*. During this process the paramutated allele is converted into a paramutagenic allele. This kind of silencing is also transgenerational (Castel and Martienssen 2013).

3.4 Detection

As the technique of RdDM results in changes in gene function that are mitotically and/or meiotically heritable and do not entail a change in DNA sequence, detection/screening of the DNA sequence will not be constructive. Epigenetic effects refer to heritable changes in the function of genes that cannot be reversed by changing the DNA sequence.

Plants that have been altered by means of the RdDM-technique can show different patterns in DNA-methylation of promoters, which results in expression or suppression of a gene or, at another level, in histone

modification. Genes with strongly methylated promoters are generally inactive and, *vice versa*, promoters which are not methylated can express their gene-products unhampered.

DNA methylation in detail is a covalent methylation at a cytosine (C) at position 5 (5-methylcytosine, 5mC) and may currently be detected by a large variation of techniques for determining DNA methylation patterns and profiles. In general, genomic DNA is hydrolysed, followed by specific detection.

There are currently three main approaches to distinguish between 5mC and unmethylated cytosine in DNA:

-bisulfite conversion

-restriction enzymes

-affinity enrichment

A versatile method for detection of DNA methylation is sequencing *via* bisulfite conversion. DNA to be determined is incubated with sodium bisulfite, which deaminates unmethylated cytosine to uracil (U). Methylated cytosine is not involved in the reaction and will remain unchanged. This way, the epigenetic modification turns into a difference in DNA sequence. This change in DNA sequence (C->U) can be detected using already existing methods, including PCR amplification followed by DNA sequencing. The use of bisulfite-converted DNA for methylation analysis is surpassing every other methodology (Gupta et al., 2010) and can also be transformed into high-throughput assays.

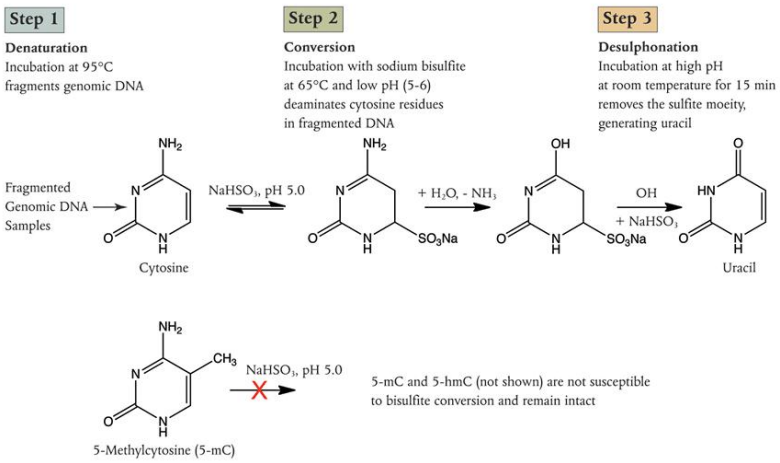

Figure 2: Bisulfite conversion (www.neb.com)

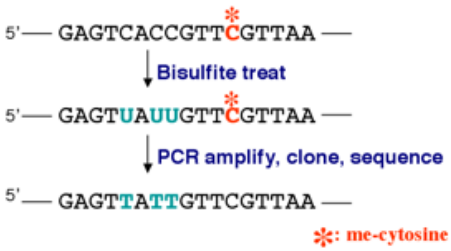

Figure 3: Genome after bisulfite treatment (www.alphabiolab.com)

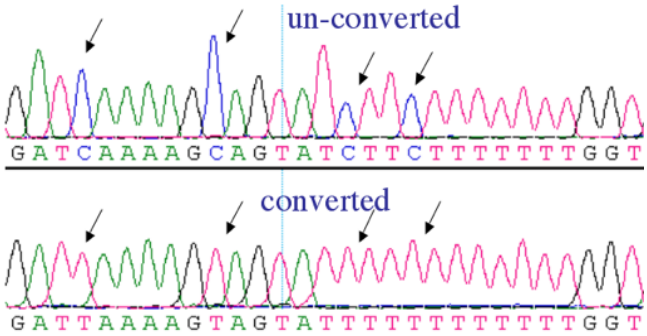

Figure 4: Result of bisulfite sequencing (http://www.alphabiolab.com/page2/page2.html)

After bisulfite treatment, the resulting genome largely consists of only three nucleotides (A, G and T, see figure 3), which elicits technical and analytical challenges.

A newly identified covalent DNA modification is 5-hydroxymethylcytosine whose epigenetic relevance is not very clear yet. This modification possibly marks a step towards DNA demethylation. Methodologies that can distinguish 5-hydroxymethylcytosine from 5-methylcytosine at a genome-wide scale are in development.

Another approach is based on the ability of certain restriction enzymes to distinguish methylated DNA from unmethylated DNA. Various enzyme-based genome-scale DNA methylation approaches exist, such as restriction landmark genome scanning (RLGS), or PCR-based methods such as methylation-sensitive arbitrarily primed PCR (MS-AP-PCR), methylation-sensitive representational difference analysis or amplification of inter-methylated sites (AIMS) (Laird 2010). In addition to bisulphite sequencing Haque et al (2007) controlled methylation of transgene Coat protein gene of sweet potato in *Nicotiana benthamiana* by methylation-sensitive restriction endonuclease digestion. Genomic DNA was digested with three units of methylation-sensitive restriction enzymes (*Aci*I, *Hpa*II, *Msp*I and *Hha*I) in combination with *Eco*RV. *Eco*RV had no recognition sites within the transgene und was used to facilitate complete digestion of the genomic DNA. Digested DNA was then subjected to transgene-specific PCR amplification. PCR-products are afterwards visualized on a gel matrix. Bisulfite sequencing confirmed the results.

Other technologies combine enzymatic methods with microarray-based analyses (Gupta et al. 2010), and commercially available platforms enable genome-scale DNA methylation analysis, for example NimbleGen (Roche), Affymetrix, Agilent and Illumina.

Another approach to detect methylated DNA (meDNA) is by taking advantage of the affinity of certain proteins that specifically recognise and bind to 5-methylcytosin (5mC). This interaction is the base for enrichment of 5mC-containing regions via ChIP-like technologies. Antibodies that are specific for 5mC or antibodies which have a high affinity to methyl binding proteins are involved in this analytical technique. Butcher et al. (2010) used the affinity of meDNA binding domain proteins with anti-meC antibodies as specific reaction.

Antibody-based enrichment is also an option for the analysis of 5hmC because 5mC antibodies do not recognize 5hmC.

Alternatively, at another level, RNAs can also be used to provide information on the epigenetic change of a promoter region, as they are the logical consequence thereof.

RNAs are studied by regular RNA technology for isolation and enrichment (Miki et al. 2008, Febres et al. 2008, Cigan et al. 2005). The expression of (trans-)genes is usually analysed by qRT-PCR (Zhang et al. 2013, Miki et al. 2008). Zhang et al. (2013) for example showed expression-levels in transgenic rice lines by using RNA blot hybridisation (northern blot analysis).

RNA-sequencing, in order to characterise and identify the RNAs, was often assigned to specialised institutes (Zhang et al. 2013). Briefly, mRNA is enriched and interrupted to short fragments, then cDNA is synthesised using the mRNA fragments as templates. Afterwards, sequencing adaptors are ligated to the fragments, PCR amplification is done and eventually sequencing analysis is carried out *via* Illumina HiSeq 2000. RNA sequencing, or second generation sequencing, with focus on the small RNA complement of cells for small RNA profiling is now more or less routine.

3.5 Traceability and variety registration

Unequivocal detection of the use of RdDM is not possible, which does not allow applying currently existing labelling regimes foreseen for GMOs (according to Regulation (EC) No 1829/2003, involving an unequivocal detection method, and reference material). However, when the information on the use of RdDM was provided during the variety registration process the general traceability as foreseen for food, feed and seed as discussed in AGES (2012) applies. Currently it is not compulsory to declare the use of a specific technique when applying for variety registration. If traceability was to be ensured by continuous labelling the regulatory measures have to be adopted accordingly.

3.6 Risk assessment

In case that templates for dsRNA are intended to be inserted into the plant genomes, which has been the method of choice in most of the current RdDM experiments, the resulting plant by definition constitutes a "GM plant", and the risk assessment procedures should be according to current guidance documents and regulation regarding GMOs (EFSA 2011a; EC 2013). In addition, risks associated with transfection with synthetic RNA molecules have to be considered.

3.6.1 Transformation method

For RdDM the same standard transformation protocols (*i.e.* biolistic transformation and transformation with *Agrobacterium tumefaciens*) are applied as used for the production of conventional transgenic organisms.

The principles of conventional plant transformation methods were extensively discussed in our previous report (AGES 2012), and it was identified that the same procedure related risks already identified for conventional plant transformation pertain whenever these methods are applied. Here, only the most important aspects related to potential risks are highlighted.

Transfer of superfluous vector backbone DNA into plant genomes is to be avoided according to current EFSA guidance (EFSA 2006; EFSA 2011a). However, in many cases vector backbone sequences are transferred. In this context, it has to be taken into consideration that 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.

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 (somaclonal variation), and the primary transformants may harbour unknown variations. These are not due to the transformation process itself but result from the tissue culture techniques, and 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 programs, similar to traditional strategies.

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). Stringent selection processes in *in vitro* cell culture systems are required, which exert substantial stress on the transformed cells and may induce unintended effects in the plant metabolism (Filipecki and Malepszy 2006).

The ***Agrobacterium*-mediated transformation** process is mainly based on tissue culture techniques and is currently the method of choice for the genetic modification of plant cells. Its major advantages 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).

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.

One of the major drawbacks of *Agrobacterium*-mediated transformation is that due to the imprecise nature of T-DNA integration at the left border site (nicking of the left border T-DNA) frequently non-T-DNA sequences from the vector backbone are integrated into the plant genome (Petti et al. 2009). This phenomenon has been shown to occur frequently in virtually all plant species amenable to this transformation technique (De Buck et al. 2000; Lange et al. 2006), and may lead to microhomology between a transgenic plant and a microorganism.

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. Moreover, 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 (Petti et al. 2009). 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). Preference for T-DNA integration in highly transcribed regions has been shown, and the locus may influence insert stability. Interactions with different genetic backgrounds are possible and may result in unexpected consequences. At the same time, the dynamic nature of plant genomes makes them prone to unintended effects unrelated to the target traits.

3.6.2 Molecular characterisation

Genetically modified plants produced by RdDM differ from GMOs constructed using alternate techniques by the fact that the final product should not contain the transgenic insert and other foreign DNA sequences. Ideally, RdDM plants are free from transformation mediated DNA sequence alterations. The phenotype of the final product solely relies on epigenetically inherited changes in the chromosomal methylation pattern. Considering this it is clear that certain aspects of the molecular characterisation recommended by EFSA (EFSA 2011a) and enforced legally by the European Commission for transgene-containing plants are not applicable for the final commercialised product (e.g. providing sequence information of the transgenic insert from the final product).

When stable insertion of templates for dsRNA into the plant genomes is foreseen, these plants are transgenic and should be checked according to the procedures in the EFSA guidance for conventionally produced transgenic plants (EFSA 2011a). By this, unintended and/or pleiotropic effects in the plant line finally commercialised can be minimized. Sequence data are essential to provide evidence that the insertion locus is free from deleterious mutations in the final product.

Concerning the molecular characterisation, the applicant should provide the following information (modified from the current EFSA guideline (EFSA 2011a):

1) Description of the methods used for the genetic modification
2) Source and characterization of nucleic acid used for transformation
3) Nature and source of vector(s) used including nucleotide sequences intended for insertion
4) General description of the trait(s) and characteristics which have been introduced or modified
5) Information on the sequences actually inserted/deleted or altered. The copy number of intact and fragmented inserts should be established.
6) Information on the expression of the inserted/modified sequence; including potential alterations to the flanking regions to exclude:
   i) the formation of potential fusion proteins
   ii) the unintended expression of a host promoter in close proximity of the insert locus and
   iii) the expression of a host gene as read-through product from the insert derived promoter
7) Genetic stability of the inserted/modified sequence and phenotypic stability of the GM plant

The final commercial product is not supposed to contain any process related aberrations in the plant genomic sequences (DNA mutations, deletions, insertions or other sequence rearrangements), and should not contain any transgenic inserts, fragments thereof or transformation vector backbone sequences information. Absence of vector, insert and additional superfluous DNA sequences in the final product must be demonstrated (Southern blot analysis). The insertion locus and the associated flanking regions identified during the pre-commercial phase of the plant development process should be checked for sequence rearrangements in the commercialised product by DNA sequencing.

Unintended effects

The alteration in the methylation pattern of the targeted DNA (regulatory) region should be characterised. Additionally, information about actually observed intended and unintended changes in the plant genome methylation pattern should be provided.

To avoid unintended effects and assess a potential impact on non-target organisms a thorough *in silico* analysis of the involved RNA sequences is recommended (Ramesh 2013). Upon transformation of plant cells with gene cassettes coding for dsRNA secondary siRNAs with altered target sequence specificity may be formed (Dillin 2003; Sijen et al. 2007). The newly generated siRNAs may have an effect on non-target genes or non-target organisms (Baum et al. 2007; Gordon and Waterhouse 2007).

Plant-derived siRNAs are mobile. The gene silencing effect is transported to neighbouring cells *via* plasmodesmata and *via* the phloem throughout the whole plant (Ramesh 2013). A distribution of the silencing signal to non-target tissue is inevitable. Tissue-specific gene silencing is impracticable using siRNA mediated approaches (Molnar et al. 2010; Molnar et al. 2011).

The establishment of minimal thresholds for necessary sequence identities, mandatory sequence lengths and optimal base pair composition is indispensable for effective gene silencing and early exclusion of affected non-target organisms (Ramesh 2013).

The RNAi induced silencing signal has the potential for amplification (Sijen et al. 2001; Molnar et al. 2011), because the involved RNA-dependant RNA polymerase produces additional dsRNA molecules from the initial template and cytosine methylation takes place in a feedback loop (Castel and Martienssen 2013). However, the extent of the signal amplification cannot be predicted or programmed, and the process is prone to produce secondary siRNAs which show altered substrate specificities (Ramesh 2013).

There may be interactions with virus-derived endogenous suppressor proteins for inhibiting the RNAi effect if the transformed plant is infected with a virus (Ramesh 2013): dsRNAs may be bound and inactivated, siRNAs may be specifically sequestrated, the whole silencing machinery may be inhibited and the spread of the silencing signal may be prevented by viral factors.

There may be also a breakdown of the silencing effect by (Ramesh 2013):

i) a mutation in the transferred dsRNA encoding transgene,
ii) off-target effects mediated by a mutated transgene, and
iii) a mutation in the target gene may help to relieve the silencing effect (e.g. no promoter methylation, virus/pest escapes).

3.6.3 Substantial equivalence

In 1995, the WHO published a report presenting the results of an expert working group that elaborated aspects and principles regarding the concept of substantial equivalence (WHO 1995). This document provided the basis for a set of guidelines and recommendations concerning the evaluation of the safety of genetically modified plants and derived products using comparative approaches, e.g. the EFSA guidance for risk assessment of food and feed from genetically modified plants (EFSA 2011a).

A comparative assessment has to consider intended as well as unintended effects. A thorough comparative assessment for evaluating intended effects is easier to perform, since intended changes relative to the conventional counterpart are measureable and can be directly addressed. Unintended effects, however, are caused by a potentially large number of parameters (pleiotropic effects, insertional mutagenesis, alterations of metabolic processes, epigenesis, changes of protein expression, etc.), and thus it is more difficult to control them or to unveil the underlying mechanisms.

At present, only a handful of scientific papers have been published describing the intentional induction of gene silencing and regulation of gene expression in plants by application of RdDM. In these study papers unintended or side effects were observed as follows:

- Spreading of DNA methylation beyond flanking regions of the targeted nucleotide sequence (Miki and Shimamoto 2008).
- Promoter DNA methylation does not necessarily induce transcriptional inactivation (RdDM) (Fischer et al. 2008).
- Inhibition effect of low temperature on RNA silencing in *Arabidopsis*, tobacco and potato (Szittya et al. 2003; Febres et al. 2008).
- Variation in silencing effects seem to depend on the generation number (Fischer et al. 2008).

According to these effects observed at RdDM plant experiments, equivalence tests between crops produced by RdDM and conventional counterparts carried out in accordance with current EFSA requirements (EFSA 2011a) seem to be pivotal. Thus, it is evident that risk assessment of crop plants produced by application of RdDM techniques needs to include a comprehensive comparative analyses based on significant comparison analyses with conventional counterparts, *i.e.* similar or closely related food or feed produced without the help of genetic modification with a well-established history of safe use (EC 2003), based on state of the art field designs and the use of powerful statistical analyses.

Such RdDM specific unintended alterations of plant genome or gene regulation can be accompanied by plant transformation effects as described in Filipecki and Malepszy (2006). This is relevant, if non-transient RdDM techniques are applied, *i.e.* templates for dsRNA are not intended to be present only transiently but inserted

into plant genomes. Furthermore important mechanisms of RdDM have not yet been revealed and not all influencing factors and potential strength and weaknesses of using this new breeding technique determined or investigated. In this respect, an appropriate compositional and agronomic risk assessment, as mentioned above, contributes to the safety of the GM plant and its derived product.

3.6.4 Toxicological and allergological risk assessment

RNA is part of the human and animal diet and, therefore, several authors propose a history of safe consumption of dsRNA and derived small RNAs (e.g. siRNA, miRNA, hpRNA) (Ivashuta et al. 2009; Parrott et al. 2010). According to Parrot et al. (2010), ingested RNA can be generally regarded as safe (GRAS). However, there is no experimental evidence available in support of this assumption (Heinemann et al. 2013). RNA in the mammalian gastro-intestinal tract is supposed to be readily degraded by pancreatic nucleases and it is assumed that there is only a remote chance for survival of RNA fragments retaining biological activity (Parrott et al. 2010). However, ribonucleotides have been shown to have an effect on the mammalian immune, hepatic and gastro-intestinal systems (Carver 1994).

Compared to ssRNAs dsRNA molecules are remarkably stable (Soukup and Breaker 1999). Uptake of dsRNA by nematode and insect digestive systems upon feeding with plant material is well documented and used as a transgenic plant protection strategy (Bakhetia et al. 2005; Huang et al. 2006; Steeves et al. 2006; Baum et al. 2007; Gheysen and Vanholme 2007; Gordon and Waterhouse 2007; Mao et al. 2007; Chen et al. 2010). Mammalian cells in cell culture systems have the capability to take up dsRNA (Amos and Kearns 1963; Schell 1967), but difficulties with delivering dsRNAs for therapeutic aims indicate barriers for the transfer of dsRNAs in intact organisms (Dykxhoorn et al. 2006).

In the mammalian system, dsRNA molecules longer than 30-bp function as potent triggers of the innate immune system and activate phosphokinase R in the sequence independent interferon pathway which shuts down cellular protein synthesis as an antiviral defence strategy (Bass 2001; Karpala et al. 2005; Chalupnikova et al. 2013). It is unclear, whether transgene encoded dsRNA molecules – usually with a fragment length between 200 and 400 bp (Ramesh 2013) – may exert similar effects upon ingestion by animal or human consumers under certain circumstances.

Transcriptome data revealed the presence of small RNA molecules in human blood and tissue, and the presence of plant-derived regulatory siRNAs has been confirmed in farm animals (Zhang et al. 2012b). Also, plant-derived miRNAs have been detected in human blood and it was shown that these RNA species silenced endogenous genes in tissue culture, mouse liver, small intestine and lung (Zhang et al. 2012a).

The peer-reviewed literature currently lacks studies which assess the safety of consuming endogenous longer dsRNAs, siRNAs, or miRNAs in human food or animal feed (Ivashuta et al. 2009). Neither the overall amounts of small RNA molecules, nor the presence of benign small RNAs in conventional plants are sufficient as evidence that all novel small RNAs will be safe in the food chain or environment (Heinemann et al. 2011).

Small RNAs exert their function via sequence-specific interactions with their target molecules. These sequence-determined activities cannot be considered as GRAS in general terms without specific supporting evidence (Heinemann et al. 2013). This view is contested by Ivashuta et al. (2009) by pointing to the observation that numerous plant-derived small RNA molecules have been demonstrated to show complete sequence complementarity to human (and other mammalian) genes without any obvious adverse effects (Ivashuta et al. 2009). However, there is no controlled epidemiological study available which would provide evidence in favour or against this assumption. Recent evidence is indicating that plant-derived small RNAs survive the passage through the mammalian gastro-intestinal tract, pass the gut barrier and have an impact on the regulation of gene expression of mammalian liver enzymes (Zhang et al. 2012a).

Thus, it remains to be nearer determined whether the naturally existing small RNAs present in plant- and animal-derived foods that make up the human diet could play an active (patho)physiological role in humans by influencing the expression of endogenous genes. The same applies to genetically modified plants based on the expression of non-coding RNA.

For RdDM plants food safety and risk assessment seems to be mainly associated with two important issues. The first being the question whether unintended effects in relation to the applied stable or transient transgenic approach have occurred and whether these are relevant for the food safety of the plant, and the second being the question whether the synthetic RNA molecules could have negative effects on animal and human health.

In conclusion, potential risks arising from application of RdDM on plants either caused by the transgenic approach itself or the introduced synthetic nucleotides need to be assessed by conducting *in vivo* animal studies that identify potential unforeseen consequences. Additionally, thorough studies of gene expression (*in vitro* and *in vivo*) with identification of the genes whose expression might be affected by specific miRNA techniques are needed.

3.6.5 Environmental risk assessment

According to a review paper considering the potential for adverse effects on the environment associated with the application of transient silencing systems (non-coding RNA) (Ramesh 2013) the following points need to be addressed:

i) Persistence of small RNA molecules,
ii) Effects on soil microbes or related viruses,
iii) Higher susceptibility to plant diseases,
iv) Alterations of siRNA and effects on host transcriptome,
v) Non-target effects on organisms ingesting plants (animals, humans),
vi) Unintended effects on molecular and cellular interactions.

The template RNA can be introduced using a transiently present vector system. In this case, specific risks may be reduced (e.g. the influence of the insertion site). The additional risks and uncertainties that are associated with the presence of newly expressed dsRNA and siRNA remain.

At present, no specific guidelines exist for the risk assessment of plants produced by application of RdDM. But taking into account current knowledge gaps and remaining uncertainties in relation to this new plant breeding technique (Ramesh 2013), the development of new approaches (e.g. transcriptome studies) and stricter requirements seems to be indicated. In this respect, EFSA notes that the ERA requires specific information sources and techniques, including experimental or theoretical methodologies, and recommendations for establishing relevant baseline information, especially for assessing long-term effects (EFSA 2010).

According to the current state of knowledge, an environmental risk assessment (ERA) for plants produced by RdDM should at least comprise the following elements, based on EFSA Guidance (EFSA 2010): Analysis of potential alterations related to persistence and invasiveness, and impacts of specific cultivation, management and harvesting techniques of the plant line under investigation; interaction of the GM plant with target organisms or non-target organisms; effects on biogeochemical processes; and effects on human and animal health.

Moreover, if plants produced by RdDM techniques were to be put on the market, it would be necessary to improve the present monitoring and surveillance systems, in order to deal with the new challenges arising from this new plant breeding technique.

3.7 Discussion

3.7.1 Drivers and constraints for the adoption of the new techniques

Advantages

RdDM allows the production of plants which do not contain foreign DNA sequences and do not carry mutations or changes in the DNA nucleotide sequence. An altered phenotype is achieved by promoter methylation, followed by the formation of heterochromatin and the subsequent silencing of the promoter-controlled gene. Methylation patterns are meiotically stable in plants and inherited to the progeny (JRC 2011).

Some of the RdDM experiments conducted indicated that no unintended effects occurred as no phenotypic changes in plants were observed. For instance, a research team at the University of Florida studied RdDM processes in transgene plants carrying parts of viral sequences of the Citrus tristeza virus (CTV). During grafting for propagation infection was established by incubation with CTV isolates. It was found that all of the transgenic plants have maintained a normal phenotype indicating that no unwanted side-effects due to the application of RdDM in grapefruit plants occurred (Febres et al. 2008). Another study which tested the ability of the CMV vector to induce promoter targeted heritable gene silencing in tomatoes observed also no phenotypic changes other than those expected from the gene silencing, indicating that the promoter-targeted silencing caused no side effect (Kanazawa et al. 2011). However, for both studies further data or tests on potential unanticipated changes at the genomic or gene regulatory level were not performed.

There are also several examples for successful induction of RdDM of transgenes or transgenic promoters in genetically modified plants and inheritance after removal of the transient inductor. The successful maintenance of CHH methylation independent of siRNA was shown in rice plants (Okano et al. 2008), though very few examples exist regarding induction and inheritance of RdDM of endogenous genes or promoters. One such example is the successful silencing of endogenous genes by transient induction of RdDM in petunia and tomato in 2011 (Kanazawa et al. 2011).

Disadvantages

Still gene silencing by RdDM is not yet fully developed and has its weaknesses. The main drawback of RdDM for the production of modified crops is the instability of the suppression of gene expression. Methylation patterns are not inherited uniformly to all progeny and, moreover, the silencing effect fades out and is lost after an indeterminate number of generations. The intensity of the silencing is usually proportional to the degree of methylation (Fischer et al. 2008), but there are exceptions (Okano et al. 2008). Gene silencing usually does not lead to a complete knock-out of gene function. The silencing effect can vary by more than two orders of magnitude in the F1 generation and the differences between individual plants can become more pronounced in the succeeding generations (Fischer et al. 2008). There is still a substantial lack of knowledge on promoter functions in the plant genome: Not all promoter sequences are equally responsive to methylation and it is not unequivocally clear which sequences exactly are involved in up- and down-regulation of target genes.

Diverse factors still unknown or unrevealed play a role in the induction of precise RdDM processes in plants. Therefore the precision of transgenic approach to date is limited. Despite direct correlation between transgene copy number and the silencing effect was indicated in previous studies, a research study of RdDM processes in transgene grapefruit could not establish this association (Febres et al. 2008).

The most important but not completely and functionally characterised factors seem to be transgenic localisation, intermediate processes, interactions with different proteins, competing machineries and the integration locus (Dalakouras et al. 2011). At least in the case of hairpin transcripts, an important factor for efficient triggering of RdDM seems to be the integration locus of a transgene. Another important parameter is the potential interaction with various proteins of competing machineries (Dalakouras et al. 2011).

There seems to be evidence that also environmental factors (e.g. temperature) can interfere with the silencing pathway in certain plant species (Febres et al. 2008). Furthermore, differences in RdDM regulation could also depend on the plant species used, as, for example, a silencing experiment targeting seven endogenous rice gene promoters indicated that some endogenous promoters in petunia and maize could be more sensitive to siRNA signals than the endogenous rice genes examined. These assumptions arise from the fact that successful gene silencing was shown for the transgenic *35S::GFP* gene but not for the seven endogenous, with the exception of partial silencing of the *Se5* gene (Okano et al. 2008). These results suggest that RdDM is highly influenced by gene features as well as plant specific characteristics. If promoters of regulatory proteins (e.g. transcription factors) are targeted, pleiotropic effects are to be expected. Such unintended effects may be intensified if the produced dsRNAs are targeted to conserved sequence regions.

Interestingly, the silencing effect and promoter DNA methylation are also not always correlated. In an RNAi study with *Arabidopsis thaliana* silencing was induced by a transgenic *ProNOS* system. One target transgene showed substantial DNA methylation but only limited inactivation. This result shows that apparently promoter methylation is required but not sufficient for transcriptional inactivation (Fischer et al. 2008).

Although silencing effects even after removal of transient vector systems have been reported (Kanazawa et al. 2011), the experiments with the transgenic *ProNOS* systems in *A. thaliana* are an example for reactivation of gene expression and the loss of methylation (at least at asymmetric cytosine) and diminishing of levels of silencing after removal of the transgenic *ProNOS* system (Fischer et al. 2008). Such results indicate that stable and high RdDM levels after removal of transient induction systems can be a difficult task to achieve.

Another critical point arises from the fact that the variation in silencing was found to be higher in progressive generations than in the F1 plants. So, the variation among the F3 plants doubly homozygous for target and silencer transgenes was 140-fold for KKchr1-10HH, 230-fold for KKchr2-3HH and 10-fold for KKchr3-3HH, surpassing the variation for the control plants homozygous for the target transgenes alone, as well as the variation for the F1 plants hemizygous for target and silencer transgene (Fischer et al. 2008). Thus, variation among individual plants may become more prominent in progressive generations.

Last but not least, spreading of DNA methylation beyond flanking regions of the targeted nucleotide sequence can be a potential disadvantage. Spreading of methylation may lead to silencing of non-targeted DNA and is considered an unwanted effect of vector-induced RdDM which seems to be difficult to control. Silencing experiments in rice by suppressing the *OsRac7* gene, for instance, led to the discovery that *de novo* methylation could spread both 5' and 3' from the RNAi target region. However, suppressing the *OsRac6* gene led to extended *de novo* methylation only in the 3' direction (Miki and Shimamoto 2008). RNA silencing targeting the transgenic *ProNOS* system in *Arabidopsis thaliana* plants however showed no evidence of spreading of DNA methylation into adjacent sequences (Fischer et al. 2008).

Actually, not many studies tested for the potential of spreading of *de novo* DNA methylation during vector induced RdDM in plants, and therefore data are insufficient for estimation of probabilities for the occurrence of this secondary effect.

The different RdDM studies available usually do not expand on potential negative effects as effects of spreading of DNA methylation, and most of the study authors only refer to the observed phenotypic equivalence but do not present tests for detecting changes at the genomic level.

If RdDM wishes to become a broadly accepted procedure in plant gene technology for the production of commercially released crops, the durability of the silencing effect has to be improved and the dsRNA encoding transgene cassettes have to be optimized to achieve better and more reliable results. It will be also necessary to enhance our understanding of promoter function in plant genomes and to elucidate the role of additional DNA sequences which are involved in the regulation of plant gene expression.

It will be also difficult to obtain a strict tissue specific gene silencing since the involved 24-nt siRNA molecules are mobile and can be transferred from cell to cell and *via* the phloem throughout the whole plant (Chen 2010; Molnar et al. 2010). Moreover, the reasons for potential spreading of DNA methylation needs to be further elucidated and sufficient information derived for the understanding how to avoid extension of methylation beyond the target site.

With respect to potential risks arising from introduced regulatory-RNA molecules, there are currently no peer-reviewed studies available which would evaluate the safety of plant derived dsRNA, siRNA or miRNAs in the human gastrointestinal tract or in animal feed (Heinemann et al. 2013). However, plant-derived miRNAs have been detected in human blood and it was shown that these RNA species silenced endogenous genes in tissue culture, mouse liver, small intestine and lung (Zhang et al. 2012a). Although a history of safe consumption for these RNA species is proposed (Parrott et al. 2010) no experimental data in support of this statement are available (Heinemann et al. 2013). Double stranded RNA molecules are remarkably stable compared to ssRNAs (Soukup and Breaker 1999). Uptake of dsRNA by nematode and insect digestive systems upon feeding with plant material is well documented and used as a transgenic plant protection strategy (Bakhetia et al. 2005; Huang et al. 2006; Steeves et al. 2006; Baum et al. 2007; Gheysen and Vanholme 2007; Gordon and Waterhouse 2007; Mao et al. 2007; Chen et al. 2010).

In the mammalian system, dsRNA molecules longer than 30 bp function as potent triggers of the innate immune system and activate phosphokinase R in the sequence independent interferon pathway which shuts down cellular protein synthesis as an antiviral defence strategy (Bass 2001; Karpala et al. 2005; Chalupnikova et al. 2013). It is unclear whether transgene encoded dsRNA molecules – usually comprising of fragment lengths between 200 and 400 bp – may exert similar effects upon ingestion by animal or human consumers.

4 Reverse breeding

4.1 Introduction and definition

Meiotic recombination is suppressed in a selected individual heterozygous plant chosen for its elite quality. Gametes are directly converted into adult plants, which after chromosome doubling are used as homozygous parental lines. Upon crossing, the lines can reconstitute the original genetic composition of the selected heterozygous plant from which they were derived (NTWG 2011). The main objective of reverse breeding is to generate homozygous parental lines (complementing parents) that can be mated to recreate a desired heterozygous genotype (*i.e.* the initial hybrid; Wijnker et al. 2012).

The following steps for producing crop plants are necessary (see NTWG 2011; JRC 2011):

1. An elite heterozygous line is selected
2. Meiotic recombination is suppressed (through RNA interference, RNAi)
3. Cells that do not contain the transgene are regenerated into homozygous, doubled haploid plants
4. Parental lines are selected – only non-transgenic plants are selected
5. The desired heterozygous genotype is obtained via crossing
6. The final heterozygous plants are non-transgenic.

Generally, meiosis may be suppressed by chemical or physical means or environmental factors. Recombination can be prevented or suppressed by various means, in particular through diverse dominant transgenic approaches but also by treatment with a chemical (Dirks et al. 2004). By dominantly suppressing genes required for meiotic recombination by RNA interference (RNAi) or comparable gene silencing techniques (siRNAs, dominant-negative mutations, VIGS, grafting, or suppression by chemicals), gametes without crossover recombination are produced from the desired heterozygote. Genes involved in the meiotic recombination process are mostly silenced through GM techniques (transgenesis). However, the inducer transgene is segregated out during further breeding and is therefore not present in the final product (negative segregant, JRC 2012). No changes are foreseen in the genome of the selected non-GM offspring (JRC 2011), and the offspring of the selected parental lines would not carry any additional genomic change (Lusser et al. 2012). For example, a dominant RNAi approach renders 50% of the offspring semi-sterile as they carry the transgene (Wijnker et al. 2012).

4.2 Applications

The technique does not aim at specific traits. Reverse breeding is used to preserve elite genotypes, for parental line substitution, and to generate chromosome substitution lines. Non-recombinant chromosomes are fixed in doubled haploid lines, which are expected to be perfectly complementing.

The concept starts from the “end product”, *i.e.* an elite hybrid. “Immortal” lines are generated that allow the indefinite propagation of uncharacterized heterozygous plants without knowledge of their provenance (Wijnker et al. 2012). Meiotic recombination is suppressed and gametes with various combinations of the intact chromosomes from either parent are formed. Rare viable gametes can be turned into adult plants by producing haploids and subsequently doubled haploids (Chan 2010). By this, two inbred lines may be created from an elite hybrid. Upon crossing the hybrid genotype is recreated and hybrid vigour is fixed.

Creation of chromosome substitution lines is possible. A chromosome substitution line contains one or more chromosomes from one parent in the genetic background of the other parent (JRC 2011). Chromosomes can be shuffled in all possible combinations when a single chromosome from one inbred is transferred into the

background of a different inbred parent. Such lines may be used for various purposes, like trait mapping or targeted inbreeding (Nadeau et al. 2000). Substitution lines has the potential to produce the same variety with potentially better reproducibility, therefore it is possible to overcome seed production problems in some crops (e.g. cauliflower) which could hinder commercialization of hybrid varieties (JRC 2011).

Heterozygous genomes may be fixed by this method. Furthermore, it can be used for marker-assisted selection (MAS) targeting individual chromosomes (Kumar et al. 2011).

4.2.1 Plants, breeding goals and anticipated developments

To date, one experimental paper describing a reverse breeding approach has been published (Wijnker et al. 2012). In this work, RNA interference (RNAi) was used to knock down the function of the meiotic recombination protein DMC1 (DISRUPTED MEIOTIC cDNA1). By this, a range of fertility genotypes was obtained, including the semi-sterile phenotype of *A. thaliana dmc*1 mutants.

Generally, the method is applicable to all species with a chromosome number of 12 and less, and for which a doubled haploid technique, preferably microspore culture, is available. According to Dirks et al. (2004) the method may directly be used for maize, rice, onion, cucumber, sugar beet, *Brassica spp*. and eggplant. It may also be applied for chickpea (Kumar et al. 2011). In view of continuously evolving research and state-of-the art it may be expected that the list expands constantly.

Doubled haploid techniques (DH) are available and successfully used for commercial production of asparagus, barley, eggplant, melon, pepper, rice, tobacco, triticale, wheat and brassica species like *Brassica juncea* and *Brassica napus*. More than 290 varieties have already been released (Murovec and Bohanec 2012). The technique could be of interest for many agricultural and vegetable species with small chromosome numbers ($n \leq 12$) that have no polyploidy background and an already successful implementation of doubled haploid production. The potential application is the optimisation of hybrid breeding and hybrid seed production. The application will, however, also depend on whether there are already well reproducible parental lines for the production of hybrids available, like it is the case for maize.

4.3 Basic principles

Recombination is crucial for chromosome pairing and is initiated by double strand breaks (Osman et al. 2011). Crossovers occur between two homologous chromatids and provide physical connection in partnership with sister chromatid cohesion (Crismani et al. 2013). A correct meiotic division requires at least one crossover per pair of homologues (obligate crossover). In the absence of crossovers there is no link between homologous sister chromatids and thus homologous chromosomes. Consequently, chromosomes segregate randomly, leading to a large number of unviable gametes.

4.4 Detection

Reverse breeding allows the breeder to reconstruct parents of a novel proprietary breeding line without having to go through the tedious back-crossing and selection process.

Transgenes are only used in intermediate breeding steps when producing homozygous parental lines from heterozygous plants by ruling out meiotic recombination. This essential step, the suppression of recombination, is achieved by introducing an RNAi construct that suppresses the action of one of the known recombination genes to a heterozygous parent line. The knockdown of the essential gene expression can be achieved by targeting genes using RNAi or siRNAs, which will result in predominantly post-transcriptional gene silencing. In crops in which stable transformation is difficult or impossible to achieve other techniques like VIGS can be used to induct PRGS. Alternatively, target genes may be silenced by silencing molecules delivered

by graft transmission (Dirks 2009). This genetically engineered parent is then crossed with a non-GM but then identical heterozygous second parent line. Half of the progeny will not contain this RNAi-producing transgene any more. These thus obtained achiasmatic gametes (gametes where cross overs did not occur) are selected and regenerated into doubled haploid plants, which upon selection and crossing can be used as hybrids like the primary hybrid. Consequently these products no longer contain transgenes (Lammerts Van Bueren et al. 2007).

The resulting end product of reverse breeding cannot be differentiated from GM varieties. Detection based on DNA screening will not reveal foreign DNA sequences. Nonetheless RNAi constructs can be detected in an intermediate breeding step aiming at generating achiasmatic gametes. According to Mendelian's rules half of them will contain the transgene construct. As products at this stage of breeding are not ready for marketing, and the majority of immature pollen is not viable, the control of this intermediate step will not be a relevant field of testing.

Neither other methods based on protein-detection nor expression analysis by transcriptomic or metabolomics approaches will reveal a pivotal information on the use of a transgene which was involved in the breeding process. The resulting hybrid is genetically identical to the initial hybrid which should be reconstructed in that way. As neither a transgene nor a product of the transgene is present in these plants, they are not recognizable or detectable.

Wijnker et al. (2012) created homozygous parental lines from a vigorous hybrid individual of *Arabidopsis thaliana*. DMC1, the gene which encodes the meiotic recombination protein DISRUPTED MEIOTIC cDNA1, was silenced in order to create the original hybrid again by intercrossing. They used RNAi to knock down the function of the RecA homolog DMC1, a meiosis-specific recombinase essential for the formation of crossovers. The effective knockdown of the meiotic recombination pathway was confirmed by transcriptional analysis of the RNAi cassette and the endogenous DMC1 gene. Progeny which was free of these of transgenic constructs was used for further activity. For consequence, the so yielded reverse-breeding haploids and their derived doubled haploids exactly reconstitute the original vigorous hybrid. This can also be confirmed by verifying the complete absence of recombination, consistent with cytological analyses, which has been shown.

As this case shows the created doubled haploids are identical to the initial hybrid, the end product finally does not differ in any way from its origin and consequently cannot be differenced by any means of analytical technique.

Wijnker et al. (2012) also mentioned that four additional reverse-breeding haploids did show crossovers, which might have resulted from accidental cross pollination or from incomplete silencing of DMC1 by the RNAi construct. In order to verify the plant's authenticity attention can be drawn to the detection of usually used RNAi constructs or the absence of known genes involved in meiotic recombination.

4.5 Traceability and variety registration

As discussed above, reverse breeding by definition does not result in any changes to the genome. Lines and hybrids resulting from this plant breeding technique are neither genotypically nor phenotypically distinguishable from traditionally bred ones. Changes to the genome resulting from the transformation process cannot be excluded. As such unintended effects should be avoided they may in principle not serve as a basis to detect and trace the application of the technique. If plants developed by reverse breeding were classified as GMO all relevant regulatory measures would apply. However, due to the mentioned constraints concerning detection, traceability and labelling as currently foreseen for GMOs according to Regulation (EC) No 1829/2003 cannot be applied for plants developed through reverse breeding. A variety may be marked as "resulting from reverse breeding" if the use of the technique is disclosed during the variety registration

process. As such declaration is currently not foreseen the relevant regulatory measures should be amended accordingly if traceability was to be ensured.

Given the declaration of the technique, the use of reverse breeding may be traced back based on the general traceability provisions for food, feed, and seed. The relevant regulatory measures have been extensively discussed in a previous report (AGES 2012). Commonly, traceability of commodities and products is ensured by documentation throughout the supply chains, starting from variety registration and basic seed production.

In conclusion, traceability of relevant plants and their products could be foreseen based on the general regulatory framework on seed, food and feed, relying on continuous documentation from the seed to primary production up to the final product. In this case declaration of the modification technique employed in the application documents may be a mandatory for variety registration.

4.6 Risk assessment

4.6.1 Transformation methods

The transformation methods to produce the intermediate lines are generally the same as in transgenesis. A dominant approach has to be used to suppress the targeted meiosis gene; currently RNAi is described as the method of choice (Wijnker et al. 2012). By using this approach well-conserved meiotic genes can be targeted across a number of different species, optimally with a single construct, which makes RNAi more attractive compared to other methods suitable to achieve the desired suppression effect (listed in Dirks 2011).

4.6.2 Molecular characterisation

The intermediate plant is clearly transgenic, produced by standard transformation methods using standard vectors. Thus, the intermediate plant should be thoroughly analysed, as the same procedure related risks apply – for details see Chapter 3.6.1, this report, and AGES (2012). The molecular analysis of the intermediate plant provides an indication of potential unintended effects in the negative segregant that result from the transformation process (EFSA 2011b).

The G02 programme launched by the UK Food Standards Agency (http://www.food.gov.uk/multimedia/pdfs/g02report) examined the use of transcriptomic, proteomic and metabolomic techniques in a number of different plant species and assessed the potential utility of “omics” approaches in comparative (GMO/non-GMO) analysis. In this frame, changes in the transcriptome of GM barley compared with that of negative (null) segregants by means of microarrays and SAGE approaches (series analysis of gene expression) were examined. SAGE revealed that 58 genes show a 5-fold difference in abundance between GM and negative (null) segregant lines. Microarrays detected approximately 8% of genes showing different levels of expression in a different GM line.

Genetic stability is not a relevant element of the risk assessment of plants resulting from reverse breeding. The suppression of meiosis in the primary transformants is sufficient to achieve the desired effect. The characteristic needs to be available during a defined timepoint in the breeding scheme but is not desired at later stages of product development and during commercialisation.

4.6.3 Substantial equivalence

From the present perspective, there is no reason that the risk assessment for plants produced by reverse breeding should exclude tests for substantial equivalence. Reverse breeding is a plant breeding technique that is not applied for trait development but for the creation of elite hybrids, more specifically, the construction of homozygous parental lines that, when mated, perfectly reconstitute the selected heterozygous genotype

(Wijnker et al. 2012). Reverse breeding includes a step at which suppression of crossover formation in a parental plant (an F1 elite plant) is implemented. From current scientific knowledge (one experimental paper exists) (Wijnker et al. 2012), it can be concluded that this step uses genetic transformation mechanisms similar to those known from standard genetically modified plants with the possibility to create negative effects due to unintended genetic and epigenetic variations.

In cases where appropriate comparators are not available (e.g. where significant compositional changes have been targeted) the EFSA GMO Panel considers to carry out a comprehensive safety/nutritional assessment on the GM plant *per se* (EFSA 2011a). However, the F1 elite plants can be used as conventional counterpart as defined by EFSA, and thus the problem that a conventional counterpart is not available and the approach of substantial equivalence cannot be employed as mentioned by EFSA Guidance (EFSA 2011a; EFSA 2011b) does not exist for reverse breeding.

Field tests and comparative analysis should be comprehensively and thoroughly performed in order to enable a reliable estimation of any potential difference in composition or phenotypic characteristics. Additionally, the comparative data should be checked against field trial information derived from the cultivation and selection process of doubled haploid plants.

4.6.4 Toxicological and allergological risk assessment

The toxicological and allergological risk assessment addresses the impact of any biologically relevant change in the GM plant and/or derived food and feed resulting from the genetic modification. Toxicological risk assessment demonstrates that the intended effect(s) of the genetic modification and any unintended effect(s) which have been identified or assumed to have occurred have no adverse effects on human and animal health. Furthermore, assessment of possible allergenicity of newly expressed proteins and of the whole GM plant, and adjuvant activity, is required for GM plants (EFSA 2011a).

Possible unintended effects in relation to reverse breeding and negative segregants in general were taken into consideration by an expert panel within a workshop hosted by Food Standards Australia New Zealand in 2012 (http://www.foodstandards.gov.au/publications/Pages/New-plant-breeding-techniques-workshop-report.aspx) leading to the results that the panel did not consider they could reach firm conclusions because insufficient technical detail was available on how transgene-free end products are produced, as well as the reliability of the process overall.

It was further concluded that, even though there should not be any particular hazards associated with the GM component of the technique, it would be helpful to develop some criteria for distinguishing techniques such as reverse breeding from those where the final food-producing lines are clearly GM and also for ensuring that a complete barrier/genetic separation exists between the early GM breeding lines and the non-GM food-producing lines.

From the present perspective and considering remaining uncertainties with respect to unintended effects associated with negative segregants derived from genetically modified plants by application of standard transformation methods, as, for example, *Agrobacterium*-mediated transformation, in principle, most of the abovementioned issues are relevant for the toxicological and allergenic risk assessment of plants developed by the application of reverse breeding techniques.

Therefore, the current EU regulation is applicable and the risk assessment procedures should be according to current GMO guidance (EFSA 2011a; EC 2013). It is, however, clear that not all elements of the risk assessment will be applicable (expression analysis, assessment of newly expressed proteins, etc.).

4.6.5 Environmental risk assessment

Reverse breeding is of different nature as standard plant gene technology, because it does not aim to achieve to introduce new traits into crop plants but to generate chromosome substitution lines or elite genotypes.

Plants produced by reverse breeding, however, undergo genetic engineering processes or, at least, transient systems (e.g. transient DNA-transfer methods) need to be applied to induce suppression of meiotic recombination. So far, no experimental data exist as to seriously assess whether this is possible with transient vector systems.

The plants intended for commercialisation will not contain any traces of the inducing construct. The commercialised plant resulting from this plant breeding technique will not contain any foreign genes, and the occurrence of unintended effects leading to potential adverse impacts on the environment should have been included in the intermediate plant. Therefore, the elements of the ERA, including gene transfer, are not relevant for the products of reverse breeding.

According to current knowledge, it may not be expected that plants produced by application of reverse breeding should have an increased environmental risk potential compared to conventionally bred plants. As the methods to suppress meiotic recombination may vary greatly (Dirks 2011), however, at the moment it cannot be excluded that the ERA should include elements of the relevant EFSA Guidance (EFSA 2010).

4.7 Discussion

4.7.1 Drivers and constraints for the adoption of the new techniques

Advantages

Genes governing meiotic recombination are widely conserved (Wijnker et al. 2012; Mercier and Grelon 2008). Several potential candidate genes to generate inducer lines for the reverse breeding process have been identified, including the highly conserved genes SPO11, which introduces double strand breaks (DSBs), and DMC1, which is in involved in homologous strand assimilation and exchange. Wijnker et al. (2012) showed successful reverse breeding in *Arabidopsis* by silencing the DMC1 gene.

Due to conserved sequence a single cassette targeting a well-conserved meiotic gene can be used across multiple species. DMC1, for example, is present in virtually all species, in which the formation of DSBs is required for homologous pairing and synapsis (Kagawa and Kurumizaka 2010; Pradillo et al. 2012). In *Arabidopsis*, DMC1 is a single gene (Osman et al. 2011) and has been shown to have a central and fundamental function in plant meiosis (Coteau et al. 1999). In *Atdmc1* mutants, chiasmata are absent, univalent chromosomes are observed and no chromosome synapsis occurs (Osman et al. 2011). On the phenotypic level, homozygous mutants are characterised by reduced fertility, atypical male and female sporogenesis, and abnormal meiosis in pollen and megaspore mother cells (Couteau et al. 1999). The mutant produces a few viable pollen grains that are capable of fertilization. A similar phenotype was observed in the DMC1-RNAi knockdown plants (Wijnker et al. 2012), with a few balanced gametes (viable microspores) that could be cultivated to obtain homozygous doubled haploid plants.

Many genes are involved in meiosis and are potential candidates to induce reverse breeding. However, the practicability has to be proven case-by-case.

Disadvantages

Reverse breeding is a technically demanding method involving specialized techniques, like plant transformation and the production of doubled haploid plants from microspores (JRC 2011). At present, both

may have low efficiency in a given species. In addition, both techniques must be available for the plant under investigation. Applicability and success rates are species-dependent for both methods, generally limiting the number of species in which the method can be employed.

Table 1 Number of non-recombinant DHs required for reconstructing the original starting plant at different probability levels in various species

Haploid chromosome number (*x*)	Probability				Model species/crop
	0.90	0.95	0.99	1.00	
5	13	14	18	47	*Arabidopsis*
6	18	20	25	67	Spinach, corn salad
7	25	28	35	94	Cucumber
8	35	40	49	133	Onion
9	49	56	69	188	Barley, carrot, sugarbeet, most vegetable Brassicas, lettuce
10	69	79	98	266	Maize, sorgum, asparagus, cocoa
11	98	111	138	377	Banana, watermelon, celery, fennel, common bean
12	138	157	195	532	Tomato, pepper, melon, rice, egg plant

Figure 5: Number of non-recombinant DHs required

An even more fundamental restriction is that due to the fact that the production of balanced gametes depends on the plant's chromosome number (e.g. 3.25% balanced gametes in the case of *A. thaliana* with its five chromosomes; Wijnker et al. 2012). Consequently, the technique is applicable to species with a haploid chromosome number of twelve or less (*inter alia*, cucumber, onion, broccoli, cauliflower, sugar beet, maize, pea, sorghum, (water-) melon, tomato, pepper, rice, and eggplant). Thus, the method is difficult to adopt in crops with higher chromosome numbers or polyploids (e.g. soybean, canola, cotton, wheat, or potato), when unfeasibly high numbers of doubled haploids are required to find the partners that reconstruct the original heterozygous plants. In species with a haploid chromosome number of twelve, like tomato, pepper, melon, rice, and eggplant, the number of doubled haploids required would be 195, calculated on basis of probabilities (Dirks 2009; figure 5). Nevertheless, many agriculturally relevant species have less than 12 chromosomes (on a haploid basis, n=x; Dirks et al. 2004; Paterson et al. 2008; see also "Index to Plant Chromosome Numbers (IPCN)", available at http://www.tropicos.org/Project/IPCN).

The inducing step – the suppression of recombination during spore formation – has to be dominant as complete knockout of a gene by a recessive mutation would reintroduce the mutation into the offspring (Wijnker et al. 2012). Thus, dominant knock-down constructs are the method of choice.

Potential disadvantages for reverse breeding techniques arise from the fact that the generation of doubled haploid plant lines generally involves treatment with anti-mitotic drugs, such as colchicine (Forster et al. 2007) which inhibits microtubule polymerization by binding to tubulin. Colchicine is a spindle poison inducing genotoxic effects as aneuploidy on human cells (Elhajouji et al. 2011). Although colchicine is highly toxic and known to be more efficient in animal than in plant tissues it is still the most widely used doubling agent (Murovec and Bohanec 2012). However, it has been noted that most breeding companies meanwhile apply less hazardous proprietary substances for chromosome doubling which, generally, are sprayed onto the haploid seedlings in the 3- to 5-leave stage while the plants are still in a greenhouse (Geiger and Gordillo 2009).

Limitations/Disadvantages

A barrier for the use of the method is the limitation to cultures with a haploid genome of not more than 12 chromosomes. With a higher number of chromosomes it would be not workable for finding the complementary pair that reconstructs the original heterozygous plant (JRC 2011). Limitations are also given due to the difficulty in obtaining balanced gametes by chance and in which spores can be generated into double haploids (Crismani et al. 2012). Reverse breeding assumes a stable transformation or regeneration of the plant and also a possible application of DH-technique.

Few data are available in the experience of unintended changes in the genome. An incomplete suppression of meiosis could occur and lead to changes in the degrees of meiosis and recombination. In addition the presence of the RNAi or siRNA construct could have an effect on the balance of gene expression, silencing of other homologous sequences in the genome is possible. The expression level get influenced, f.i. some alteration in down-regulation and activity of gene expression could occur. The effects of an incomplete suppression and changes in expression level can also run as a natural process in plants (JRC 2001, Messmer 2011, Dirks et al. 2009).

This breeding technique does not aim at the incorporation of specific requested traits in the cultivars of species with haploid chromosome numbers in which $n < 12$. The central consideration as to the acceptance of this technique would be whether it could deliver a sustainable commercial input in optimizing seed hybrid production in species for which so far hybrid production is difficult and thus has only started, e.g. in some vegetables, barley, or rice. Hybrids will gain more importance in the future, but are subject to great commercial interest. In the coming years the technique may help to overcome restrictions in hybrid seed production but will likely remain a technique aiming at special applications.

5 Grafting

5.1 Introduction and definition

Grafting is a common technique that has been used for centuries in plant breeding. In grafting, the scion (tissues or parts) of a plant is grafted onto the rootstock of another plant. Particularly in fruit growing and viticulture, grafting has been used for many centuries. Most horticultural species cannot be reproduced from seedlings for commercial trades, therefore with grafting skipping of the juvenile stage will be achieved and is necessary to causing precocity.

Scions are grafted on rootstocks to result in uniform growth of dwarfed fruit trees ones that are more resistant to diseases and pests or other improved cultivation characteristics. Recently, an increasing number of non-woody, vegetable and some ornamental plants (particularly tomato, cucumber, eggplant, watermelon and rose plants) are grown on rootstock. (COGEM 2006, JRC 2011)

In the course of development of genetically engineered-crops, and associated environmental and economic benefits on a global scale, grafting combined with transgenesis is applied as a novel biotechnology application. A range of molecular techniques can be used to transform the rootstock and/or scion, this process is often referred to as 'transgrafting' (Haroldsen 2012a). If a GM-scion is grafted onto a non-GM rootstock, then stems, leaves, flowers, seeds and fruits will be transgenic. But the reverse process is of major interest using new plant breeding techniques: to graft a non-GM scion onto a GM rootstock. The rootstock is used for modifying or introducing traits which lead to improved characteristics of the rootstock, furthermore of the whole plant and finally, could get reflected in the "end product".

5.2 Applications

The method could be applied in all processes in which commercial grafting is already used. Commonly described techniques include butt, bench, whip, wedge, cleft, tube, splice grafting and adapted procedures. Thereby the transformation step in the rootstock is presumed, to insert the specific and desired trait. The method aims at resistance traits against diseases, in most cases soil-born fungi or bacteria, pests or to improve growing aspects, like rooting ability, nutrient and water acquisition. The desired trait is inserted via transformation of the rootstock.

The traits that are described in the primary literature can be summarized in four groups: Disease resistance, soil effects, physiological effects and herbicide tolerance. Resistance against diseases treats fungal as well as bacterial or viral diseases. These traits are the most researched in the primary literature. Plants that are researched are *Vitis vinifera*, tomato, apple, *Prunus* spp., cucumber and also model plants like *Nicotiana* spp. Soil effects especially salinity tolerance is researched in apple and watermelon, as well in *Nicotiana* spp. Physiological effects under investigation are decreased branching in pea and increased resveratrol synthesis in apple. Herbicide tolerance has been introduced into soybean.

Transformation of the rootstock can be achieved using traditional techniques like e.g. *Agrobacterium*-mediated transformation and biolistic approaches ('gene gun method') (JRC 2011). Another possibility is to trigger targeted changes in protein and gene expression in the scion, based on modified rootstock. Such changes are a result of RNA interference (RNAi), e.g. post transcriptional gene silencing (PTGS) which inhibits gene expression of targeted loci *via* methylation of cytosine. By leading to the translocation of small RNA molecules or specific proteins from the root system to the scion a GM rootstock could open up the introduction of new traits into a range of genetically distinct scions (JRC, 2011). RNA molecules (mRNAs, miRNAs, siRNAs), plastid DNA, peptides, proteins, hormones and metabolites resulting from the genetic

modification in the GM rootstock are able to pass the graft junction (Haroldsen et al. 2012a, JRC 2011, McGarry and Kragler 2013). One of the challenges in this respect is to relate these moveable elements directly to the genetic modification in the rootstock and to differentiate them from naturally occurring plant molecules and metabolites in the non-GM scion. Haroldsen et al. (2012a) compare the mobility of transgenic material with systemic acquired resistance (SAR) in plants. SAR, mediated by the plant hormone salicylic acid, demonstrates that molecules or signals can move within plants and subsequently provide resistance in anticipation of pathogen contact (Haroldsen et al. 2012). In a number of studies researchers observed the mobility of the products of the transgene together with the potential application:

Research studies demonstrating the detection of transgenic molecules:

Agüero et al. (2005): Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP (pPGIP) gene. PPGIP, under the control of the CaMV 35S promoter, activity was detected in xylem exudates of untransformed scions grafted on transgenic rootstocks expressing pPGIP. It is the first demonstration that PGIP reaches the xylem and moves through the graft union. They expect that pPGIP will also be expressed in the fruit clusters, but it was not shown in this report.

Dutt et al. (2007): Transgenic rootstock protein transmission in grapevines. GM rootstock is used to control Pierce's disease (PD), which is caused by *Xylella fastidiosa*. Production of antimicrobial peptides by the transgenic rootstock leads to a control of bacteria in the xylem sap of a non-GM scion – providing PD resistance. Non-transgenic *Vitis vinifera* 'cabernet Sauvignon' and 'Thompson Seedless' were grafted onto *Vitis vinifera* 'Thompson Seedless' rootstocks transformed *via Agrobacterium*-mediated transformation to express the Shiva-1 lytic peptide gene under the control of a 35S promoter. The presence of the peptide in xylem sap of a non-GM scion was determined.

Stegemann and Bock (2009): Exchange of genetic material between cells in plant tissue graft. In this work tobacco plants from two transgenic lines carrying different marker and reporter genes in different cellular compartments, the nucleus and the plastid, are grafted. Results demonstrate the movement of plastid genetic information across cellular barriers from rootstock to the scion and *vice versa*, but it is not clear if the genetic information was transferred as DNA fragments, as entire plastid genome or as plastid. Exchange of large sections of plastid – but not nuclear – DNA is obtained, providing a possible pathway for horizontal gene transfer.

Wang et al. (2012): *Bacillus thuringiensis* protein transfer between rootstock and scion of grafted poplar. The translocation of Bt-Cry1Ac protein, a toxin against different leaf-eating lepidopteran insects, in grafted poplar was demonstrated. The protein was transported between rootstock and scion mainly through the phloem. Migration of Bt-Cry1Ac protein in the graft union was also proved by feeding experiments.

Jiang et al. (2013): Grafting imparts glyphosate resistance in soybean. The transmission of the glyphosate resistance signal from rootstock to scion was demonstrated, suggesting that glyphosate resistance is systemically mobile across the graft junction. The glyphosate resistance trait is conferred by CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS). CP4-EPSPS was detected in a non-transgenic and glyphosate-sensitive scion (CN scion), grafted on a glyphosate-resistant (RoundupReady, RR) rootstock of soybean, but compared to a non-grafted RR plant only 0,001% of CP4-EPSPS was detected in the leaf. Due to this minimal concentration it is unlikely to have contributed significantly to the glyphosate resistance observed in transgrafted soybeans. It is proposed that amino acid systemic trafficking and/or tissue specific glyphosate resistance are possible reasons for the presented research results.

Research studies in which detection of transgenic molecules could not be established:

Gal-on et al. (2005) show in their study protection of susceptible cucumbers against soil inoculation with CFMMV (Cucumber fruit mottle tobamovirus), by grafting on a transgenic I44 rootstock, mentioned as "transgenic-rootstock mediated protection". Accumulation of the 54-kDa homologous siRNA could not be detected in I44 plants.

Hemmer et al. (2009) described researches in exchanges between rootstock and scion of a transgenic rootstock expressing GFLV (Grapevine fanleave virus) coat protein (CP) gene. It was shown that viral infection of non-transgenic scion (*Vitis vinifera* cv. Pinot Meunier) was delayed in vines grafted onto transgenic rootstock. As results transgenic-derived expression products were detected in GM rootstocks, but the systemic movement of such products (CP and *NPTII* mRNAs, and NPTII protein) was not detected, suggesting that scions remain truly wild-type.

Smolka et al. (2010): Effects of transgenic rootstocks on growth and development of non-transgenic scion cultivars in apple. *RolB* transgenic dwarfing apple rootstocks grafted with non-transgenic scion cultivars were used to investigate the function of the *rolB* gene to modify difficult-to-root rootstocks of fruit trees. Results show a significantly reduced vegetative growth of *rolB* transgenic rootstocks. Fruit quality was not explicitly affected by the GM rootstock. The *rolB* gene or its mRNA were not detectable in the scion cultivars, and no translocation was determined. This could develop the opportunity to use this model in combination with vigorous scion cultivars or to produce bonsai plants.

5.2.1 Plants and breeding goals and anticipated developments

Due to anatomical reasons grafting techniques are not successful in monocots. Thus, these methods are not available for some of the most important species in human nutrition, as e.g. corn, wheat or rice. All dicots, however, are basically approachable for grafting. As grafting means a mechanical action at the individual plant, this method is mainly applied in species with a strong emphasis on the individual plant in the production process, e.g. fruits trees (Jensen et al. 2012, Li et al. 2013, Rühmann et al. 2006, Song et al. 2013) or grape vines (Agüero et al. 2005). Furthermore, especially the perennial nature of these species makes them suitable for grafting techniques, since the successfully established scions may grow and fructify for many years. Research work with grafting has already been extended to annual crops as e.g. for some vegetable species such as tomato (Zhang et al. 2011), cucumber (Gal-On 2005) or watermelon (Yang et al. 2013). Actually grafting is a very common approach in Asia, Europe and Middle East in cucurbits and tomatoes to overcome impacts of soil-borne diseases and it is going to be evaluated for this purpose also in the USA (Davis et al. 2008). Particularly in Asia, cucumber, melons and watermelons are among plants grafted to a high percentage (Lee 2003).

In their review article about phloem-mobile signals affecting flowers McCarry et al. (2013) point out that e.g. among cucurbits one stock line may be used for other compatible species (heterografting). Among annual agricultural species Jiang et al. (2013) grafted soybeans and found that transgenic RR soybean rootstocks confer glyphosate resistance to non-transgenic soybean scions. For the cultivation of soybean with stand density of some hundred thousand plants per hectare grafting is not suitable but it may be used for breeding purposes. In breeding programmes only a few plants may be sufficient as starting material, and grafting can offer new possibilities also in annual plants depending on the breeding goal. The authors conclude that soybean is not an economically feasible crop for grafting. Nevertheless, their research provides proof of principle for the possible use of grafting to develop herbicide-resistant plants. They state that the technique could be useful for annual vegetables like tomato and cucurbits, and in addition bypasses potential gene flow.

From a breeder´s point of view grafting may be seen as a known mechanical approach to overcome difficulties in combining desired traits in one plant organism by breeding methods. By grafting advantages of the rootstock line can be combined with those of the scions. Concerning the rootstocks goals tried to be achieved by grafting technology are, e.g., increasing resistance levels against virus or fungal diseases, often soil-borne causative agents (Jensen et al. 2012, Gal-On 2005), environmental stress factors such as salinity (Li et al. 2013, Ruiz et al., 2006) and also herbicide treatment (Jiang et al. 2013).

5.3 Basic principles

Grafting makes use of a variety of cutting and inter-connection techniques to combine rootstocks and scions with different genetic background. Usually callus growth is induced, and the vascular networks (phloem and xylem) of both rootstock and scion become connected.

5.4 Detection

Grafting is mostly applied to induce disease resistance or better growth by using an appropriate GM rootstock and grafting a non-GM scion onto it. As a consequence the upper stem of the resistant graft and naturally the fruit are not being genetically modified.

Rootstocks can on the one hand be genetically modified by inserting a resistance gene into the genome, or, on the other hand by inserting genes expressing siRNA molecules which move within the whole plant and trigger DNA-methylation in desired target promoters and, as a result, silencing of the targeted gene. In this way protein production can be regulated in the upper plant without modifying the DNA sequence, and fruits may harbour novel traits without detectable changes to their DNA sequence.

Through grafting, the vascular networks (phloem and xylem) of both rootstock and scion become connected and what is mobile in the rootstock vascular network is likely to become mobile in the vascular network of the scion. To obtain an indication for a genetic modification the basis would be the possibility to detect translocated transgenic molecules in the scion or in the harvested product by suitable methods.

Transgenic proteins, hormones or siRNAs can be transported from the transgenic rootstock to the upper stem where they accumulate and cause an effect. Concerning DNA there is no current evidence that would support the movement of genomic DNA through the vascular system of a grafted plant. Nevertheless it is possible that heritable changes induced by epigenetic modification of genomic DNA may occur as a result of movement of molecules originating from the transgene in the rootstock (Haroldsen et al. 2012a). Epigenetic changes present an opportunity to endow progeny with characteristics that result from transcriptional down regulation or gene silencing without introduction of heritable transgenic DNA.

Evidence of a highly regulated and selective process involving long distance trafficking of mRNA has been demonstrated (Haroldsen et al. 2012a, Palauqui et al. 1997, Smolka et al. 2010). Diverse types of RNAs from one type of the scion (stems, leaves, floral tissues) can be detected *via* regular RNA-techniques: isolation of RNA out of plant material, enrichment, RT-PCR, RNA sequencing (RNA-Seq), etc. Given the relatively short persistence of RNA transcripts (mRNA) it is possible to anticipate any transgenic RNA in the scion tissues, fruits and other products would have been degraded as soon as removed from the plant as there is no connection to the source rootstocks that are the sites of RNA synthesis (Haroldsen et al. 2012). Small, non-coding RNAs, e.g. siRNAs, which have a strict silencing or quenching effect on gene expression, move unidirectionally from rootstock to scion (Haroldsen et al. 2012a, Palauqui et al. 1997). By means of northern hybridization the expression level of a target gene can be demonstrated representing an indirect evidence of a GM rootstock.

Proteins are macromolecules inherent in plants that can move within vascular tissues to profoundly impact normal plant growth and development. A number of investigations of transport mechanisms of foreign

proteins within host plants, especially between scion and rootstock of grafted plants, have been made (Wang et al. 2011). Wang et al. (2011b) examined the transport mechanism of foreign Bt-Cry1Ac protein in grafted transgenic poplar and analysed the lethal effect of scion leaves of the non-transgenic part of the graft on target *Clostera anachoreta* larvae, which are a natural defoliator pest of poplar or using ELISA-technique to detect the transported Bt-Cry1Ac protein itself. The protein was detected in all tissues of rootstocks and scions of the surviving grafted poplars. The content of Bt-Cry1Ac protein was highest in the phloem, which suggests that is was mainly transported in the phloem, followed by the leaves, and lowest in the xylem and pith. It can be concluded that the Bt-Cry1Ac protein decreased acropetally from the lower to the upper portion of the scion. Since information can also be transmitted between graft partners in the form of RNA, reverse transcription-polymerase chain reaction (RT-PCR) was further employed to verify that protein was translocated and not RNA. The results showed that mRNA was not translocated in poplar scions when they were grafted on GM rootstock, which provides direct evidence that information translocation was indeed through protein and not through RNA.

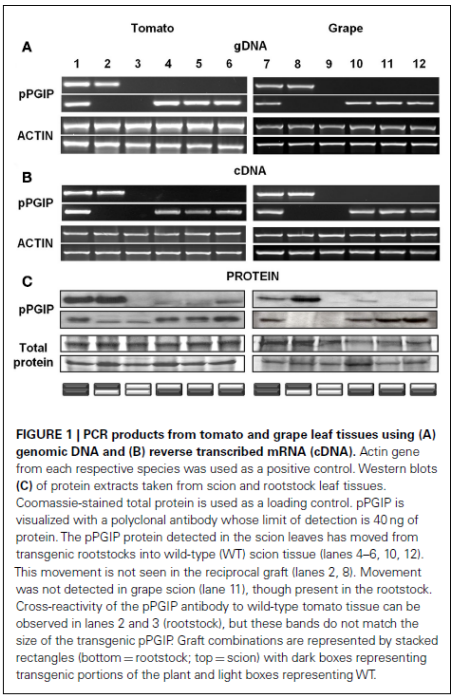

FIGURE 1 | PCR products from tomato and grape leaf tissues using (A) genomic DNA and (B) reverse transcribed mRNA (cDNA). Actin gene from each respective species was used as a positive control. Western blots **(C)** of protein extracts taken from scion and rootstock leaf tissues. Coomassie-stained total protein is used as a loading control. pPGIP is visualized with a polyclonal antibody whose limit of detection is 40 ng of protein. The pPGIP protein detected in the scion leaves has moved from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4–6, 10, 12). This movement is not seen in the reciprocal graft (lanes 2, 8). Movement was not detected in grape scion (lane 11), though present in the rootstock. Cross-reactivity of the pPGIP antibody to wild-type tomato tissue can be observed in lanes 2 and 3 (rootstock), but these bands do not match the size of the transgenic pPGIP. Graft combinations are represented by stacked rectangles (bottom = rootstock; top = scion) with dark boxes representing transgenic portions of the plant and light boxes representing WT.

Figure 6: PCR products from tomato and grape leaf tissues (Haroldsen et al. 2012a)

Proteins, like e.g. Polygalacturonases (PG), which are secreted during pathogen-infections, causing cell separation and tissue maceration can be expressed by pest and fungal pathogens (Haroldsen et al. 2012a). PGs cleave alpha-(1-4) linkages between D-galacturose residues in pectin homogalacturonan. This process can be inhibited by PG-inhibiting proteins (PGIPs) produced in fruits which are components of the defences against invasion by pathogens and pests in fruits. PGIPs expressed in rootstocks, therefore, are potential anti-pathogen proteins that could be delivered from the rootstock to the scion in transgrafted plants. Haroldsen et al. (2012a) showed that PGIPs expressed in pear reduces the effects of Pierce's Disease in grapevines, caused by the bacterium *Xylella fastidiosa*. They observed that when pear PGIP (pPGIP)-expressing transgenic plants are used as rootstocks onto which non-expressing scions are grafted, the PGIP-protein, but not the PGIP-encoding nucleic acids, are exported to the scion, crossing the graft union *via* the xylem. Also when grafting tomato plants expressing pPGIP in the rootstock the pPGIP protein was detected in scion leaves (figure 6). Thus, defence factors in roots, like here PGIP, can be made available to scions *via* grafting and can also be detected by analytical means: Western blots of protein extracts taken from scion and rootstock leaf tissues can be compared (figure 6).

Targeted analysis of specific key compounds using well-established and validated protocols provides the base for analytical detection of genetic modification in plants. There is an on-going debate over the potential value of so-called non-targeted approaches such as "omics" (transcriptomics to assess changes in the transcriptome, proteomics to analyse the constitution of the proteiome, and finally metabolomics). From such analyses knowledge on the natural variation of analytes (proteins, RNAs, small molecules) can be accumulated. The extent of such variation will be mostly driven by genotypic, environmental and crop management influences but also provides a clear benchmark against which new generations of crops can be differentiated in some cases (see G02 programme launched by the UK Food Standards Agency, http://www.food.gov.uk/multimedia/pdfs/g02report, describing the potential of "omics" technologies to analyse differences between GMO and non-GMO plants). It was shown that 2D-PAGE can be used to assess the difference in protein composition between GM and non-GM wheat. The results reveal that there are different protein profiles between GM and non-GM lines but the greatest differences arose from natural

environmental variation, rather than from the GM in question, limiting the potential of the technology to assess grafting on GM rootstock. Methods developed in the G02 programme are successful at detecting unintended changes resulting from transgene insertion into plants at the levels of RNA, proteins and metabolites. However, frequently the differences between plants grown in different environments or those between different varieties were greater than the effect of the transgene/genetic modification itself. A case-by-case approach remains the pragmatic solution.

5.5 Traceability and Variety Registration

Currently, there is no doubt that a rootstock resulting from standards transformation processes is to be considered as a GM. For these rootstocks all regulatory measures related to GMOs apply. Detection and thus traceability based on analysis, and, if applicable, quantification is possible. In a grafted plant, the rootstock will be transgenic and can be detected. The GM rootstock has to go through the standard procedures foreseen for GM lines, including risk assessment, may be labelled according to current regulations, in particular Regulation (EC) No 1829/2003, and is clearly marked as “GMO” in all relevant catalogues. Any plant grafted on GM rootstock may be labelled accordingly.

In contrast, all products (e.g. grapes and fruits) derived from the grafted plant do not necessarily harbour traces of transgenic products. By definition, they are not transgenic and do not contain inserts of foreign DNA in their genetic material. Nevertheless, gene products resulting from the transgenic rootstock may be present in the product. In most cases the unequivocal detection of these products and attribution to the origin from the genetic modification in the rootstock is not possible. Due to these reasons traceability is seriously hampered and may only be assured by continuous documentation. This documentation may be part of the general traceability measures foreseen in the food and feed supply chains (AGES 2012), as well as for propagating material.

5.6 Risk assessment

5.6.1 Transformation methods

For the transformation of the rootstocks standard transformation methods are employed, and the same procedure related risks already identified for GM plants apply. For the majority of GM rootstocks *Agrobacterium*-mediated transformation has been identified as the method of choice conferring the same risks as discussed in chapter 3.6.1.

5.6.2 Molecular characterisation

General remarks (Haroldsen et al. 2012a)

The major safety issue relates to the unintended transfer of transgene related macromolecules to tissues of the non-transgenic part of the plant which would lead to unintended aberrations in gene, protein and trait expression.

Although in a historical perspective nucleic acids were believed to be immobile and strictly cell-specific components not able to be transferred between different cells or different parts of the plant it became evident that DNA and RNA genetic or epigenetic factors have the potential to move within the plant as well as protein translocations have been confirmed. These observations made it plausible that transgenic modifications (and/or their effector molecules) introduced for instance into the rootstock may be also transferred into the non-genetically modified scion and *vice versa*. The genetically modified part of the plant does not remain to be a strictly secluded entity of the whole organism but interacts with all other parts of the plant by physical exchange of biologically active molecules – also of transgenic origin. As the transgenic part of

a grafted plant has to be thoroughly assessed for adverse effects on human and animal health and the environment according to Directive 2001/18/EC and Regulation (EC) No 1829/2003, anyway, concerning grafting a special focus has to be put on the risk assessment of the remaining, non-transgenic sections of the plant. Several aspects – as discussed below – require special attention:

Chromosomal DNA changes in non-transgenic parts of the grafted plant

So far, the crossing of the graft junction has only been demonstrated for plastid DNA (Haroldsen et al. 2012a). No movement of chromosomal DNA has been evident. Concerning risk assessment this circumstance puts a special focus on the kind of transformation protocol, which has been applied, and the localization of the transgenic insert(s). Detailed information from the applicant concerning the biolistic transformation protocol and whether plastid DNA was involved or targeted is required. *Agrobacterium*-mediated transformation, which guides the transgenic inserts to the nucleus of the plant cell, appears to provide no additional hazard for interference with the non-transgenic part of the grafted plant. Long distance transfers of transgenic organelle DNA is unlikely, however, the borders between the transgenic and non-transgenic part of the plant have to be clearly defined and non-transgenic tissue has to be checked for the presence of transgenic organelle DNA via Southern blot or PCR approaches.

mRNA translocation

Long distance translocation of mRNAs has been demonstrated to be a highly regulated and selective process in plants (Haroldsen et al. 2012b). Although transport and targeting appears to depend on specific sequences present in the 5' and 3' untranslated regions, the possibility of an unintended transfer of transgene derived mRNAs remains. Therefore, a close monitoring for the presence of transgene specific mRNAs in the non-GM part of the grafted plant RT-PCR or Northern blots is recommended.

Effects of translocation of small non-coding RNAs

Small non-coding RNAs are mobile in plants and do not restrict their gene silencing effects on single cells, where these RNAs are generated. The usually induced silencing effect is transported over long distances in the plant and the possibility of an unintended effect on gene expression also in non-transgenic parts of the grafted plant remains (Chen 2010).

In general all dsRNA and siRNA-related risks already identified for RdDM applications in plant breeding are relevant for the risk assessment of grafting (please see dsRNA- and siRNA-specific issues in chapter 3.6)

Protein translocation

Protein translocation has been demonstrated to take place over long distances and appear to be tightly regulated (Haroldsen et al. 2012a). As the presence of transgenic proteins in the non-transgenic part of the organism cannot be excluded, a tight monitoring for transgenic proteins in non-GM tissue is recommended.

Adventitious shoots from the callus (*i.e.* the border region between transgenic and non-transgenic part of the plant) or from the GM rootstock have to be closely monitored as for instance the fruits resulting from this process are transgenic.

5.6.3 Substantial equivalence

Current knowledge supports the fact that movement of nucleotides (particularly RNA), peptides and even proteins beyond the graft junction is possible (Haroldsen et al. 2012a), and so, for example, silencing signals produced from a transgenic source stock could be transmitted into a non-silenced scion (Uddin and Kim 2011).

In case of transgenic source means a rootstock transfected with synthetic RNA molecules using RdDM techniques with transient vector systems, the movement of these (small non-coding) RNA by crossing the graft

junction triggers RNA-induced developmental programs in plant scions. These may include processes that are induced unintentionally. Additionally, potential risks arise from the presence of small RNA in food and food products (e.g. fruits) derived from non-GM scions but originating from the GM rootstock. Against this background comparative assessments between these non-GM scions and conventionally produced scions (not using GM rootstocks) and food products thereof are indicated.

Regarding current EU standards concerning the use of conventional counterparts in the comparative analysis as part of the risk assessment of GM plants, the grafting technique (use of GM rootstocks) has not been evaluated. This means that a conventional counterpart has not been defined for this new breeding technique.

Per definition the conventional counterpart should be a conventional plant with a history of safe use and a genetic background as close as possible to the GM plant. In this respect, and considering that the GM rootstock usually will be in the centre of interest, which is produced to be used for grafting of different non-GM plant scions, the comparative assessment can be carried out by the comparison of the GM rootstock and its conventional counterpart. This procedure could be appropriate for testing compositional and phenotypically equivalence of the GM rootstock and a conventional, isogenic non-GM rootstock (conventional counterpart), provided that the field trials and the statistical analyses provide meaningful and reliable results.

The compositional and agronomic assessment should be in accordance with current EFSA requirements (EFSA 2011a, EFSA 2011b), so that the results are able to indicate whether there are differences (intended or unintended) or a lack of equivalence between the GM rootstock and the conventional counterpart.

5.6.4 Toxicological and allergological risk assessment

It has been shown experimentally as sensitive analytical methods have become available that nucleic acid are not contained in the cell of origin but present and functional also outside of cells where they are produced (Stegemann and Bock 2009). So, the movement of plastid DNA across cellular barriers adjacent to the graft junction, the mobility of RNA molecules and of proteins beyond the graft junction has been demonstrated (Haroldsen et al. 2012a).

In case of rootstocks developed by transgenic methods either using traditional transformation processes or new plant breeding techniques this would mean that e.g. synthetic RNA molecules or newly expressed proteins could pass the graft junction and be present in products derived from untransformed scions.

Besides comparative tests, additional studies must be performed to ensure that transgenic proteins, RNA and other mobile metabolites, as well as unintended effects due to the presence of these compounds in scion tissues that remained undetected during molecular characterisation and equivalence testing, do not have the potential for having adverse effects on humans and animals.

There are still considerable uncertainties with respect to the safety of small non-coding RNA, especially dsRNA, and the potential occurrence of unintended effects cannot be ruled out. Thus, the evaluation of the safety of plants produced by (trans-)grafting techniques should include toxicological testing of the whole food and/or feed derived from the grafted scion. The requirements, thus, are not different from the present EFSA Guidance (EFSA 2011a).

5.6.5 Environmental risk assessment

SiRNAs have been shown to exert toxic effects on target insects(Baum et al. 2007; Gordon and Waterhouse 2007) and are used as pesticides by direct feeding(Chen et al. 2010) or via application in liposomes (Whyard et al. 2009). Secondary siRNAs with altered target sites may expand the cytotoxic effect to non-target organisms (Baum et al. 2007).

Nematodes have the capability to directly take up dsRNA from the environment or via ingestion of dsRNA expressing bacteria (Tabara et al. 1998; Timmons et al. 2001). Beneficial nematodes in close proximity may suffer from exposure to modified plants expressing silencing siRNAs (Bakhetia et al. 2005).

Considering the listed elements referred to by current EFSA Guidance (EFSA 2010), the main issue for the ERA of plants produced by transgrafting (grafting non-GM scions on GM rootstocks) concerns the fact that a GM rootstock is released into the environment with possible hazards and consequences as follows:

1. Mobile metabolites may cause an unintended effect on e.g. phytoplasms, nematodes, psyllids and aphids
2. Suckers and adventitious shoots may be a source of unintended effects, in particular if they emerge from the transgenic rootstock.

Particular risks as transfer of genetic material to organisms as e.g. leaf-eating insects may arise from unwanted but mostly uncontrollable suckers that are not removed regularly. The formation of root bridges should be noted in relation to a GM rootstock-to-microorganisms (and other soil organisms) gene transfer.

As regards the transmission of mobile transgenic elements (small RNA, proteins, peptides) into non-GM scion tissues, it is noteworthy that siRNAs have been shown to exert toxic effects on target insects (Baum et al. 2007; Gordon and Waterhouse 2007) and are used as pesticides by direct feeding (Chen et al. 2010) or via application in liposomes (Whyard et al. 2009).

Moreover, nematodes have the capability to directly take up dsRNA from the environment or via ingestion of dsRNA expressing bacteria, and it should be assessed whether beneficial nematodes in close proximity may suffer from exposure to modified plants expressing silencing siRNAs.

5.7 Discussion

5.7.1 Drivers and constraints for the adoption of the new techniques

Advantages

Due to the fact that no new approaches are necessary for application of grafting (on a GM rootstock) it is possible to extend the method to further plants of agricultural importance. Nearly all fruit and nut trees grown on a commercial scale are currently grafted and recently there is also an increase of non-woody plants and vegetables for grafting methods, so using genetically engineered rootstocks is technically feasible for this industry (Haroldsen et al., 2012a). Many researches are concentrated on new application on a wider range of plants, especially for resistance against plant diseases. In addition investigations have often been derived from model plant system like *Arabidopsis* spp., tomato, and tobacco, and have to be proved if they are applicable to other plant systems. The impact of the method lays in the performance of the scion, which finally should result in increased health condition, yield and quality of “end-products”, related to the transformed trait in the rootstock. The possibility to trigger targeted changes in protein and gene expression in the scion, based on modified rootstock, open up the introduction of new traits into a range of genetically distinct scions (JRC 2011).

Disadvantages/Limitations

Of major interest is the possibility for triggering unintended changes in gene, protein and trait expression in the scion. Unwanted effects can be triggered by e.g. movements of RNA molecules (mRNAs, miRNAs, siRNAs) and/or proteins from the GM rootstock to the non-GM scion, which are not related to the targeted trait. It is unclear if these changes could result in an accumulation of genetic material in the “end product” and,

furthermore, in a release in the environment. Regarding a potential movement of DNA between rootstock and scion which could result in genome changes in the scion, Stegemann and Bock (2009) described the exchange of plastid genetic information between cells in plant tissue graft, but it is not clear if the genetic information was transferred as DNA fragments, as entire plastid genome or as plastid. There are not yet references describing changes to the coding sequence of a non-GM scion grafted onto a GM rootstock not yet.

Potential international policy differences in threshold limitations or a “zero-tolerances” for the application of new plant breeding techniques could represent difficulties for import and export regulations and therefore for labelling processes. The U.S. Department of Agriculture, U.S. Food and Drug Administration and U.S. Environmental Protection Agency coordinate regulatory frameworks, but there is no precedent for the regulation of a wild-type scion grafted on a GM rootstock. U.S. Regulation has a product-based policy for identification of genetically engineered crops; if the final product contains transgenic material it is considered as genetically engineered. In the USA products of transgrafted plants could potentially be classified as conventional and non-GM food (Haroldsen et al. 2012). Contrary, an example for a regulation of a member state in the EU is Germany: the authorities decided in 2010 that, for the time being, grapes or wine produced from transgenic rootstocks must be labelled as GMO (Heselmans 2011; Haroldsen et al.2012c).

One technical barrier could be the formation of root bridges and adventitious shoots (“suckers”), and the related possibility for transmission or exchange of genetic material. A group of specialized pathogenic bacteria (phytoplasms) are transmitted through roots bridges under experimental and natural conditions in apple orchards in Italy and Slovenia (Ciccotti et al. 2007; Ciccotti et al. 2008; Lesnik et al. 2008). It is possible that root bridges may present a feasible pathway for the local transmission of transgenic related molecules from one GM rootstock to another non-GM rootstock, in particular in old orchards. As very little is known on the frequency of root bridges in orchards, there is uncertainty on their occurrence and significance for their risk of transmission of transgenic material through root bridges.

Another problem may arise from adventitious shoots which do not present the same genotype as the scion but carry transgenic constructs like the rootstock. These shoots emerge naturally from rootstocks (regenerating from callus, the “tissue of bridge” between rootstock and scion) and may represent a source for the transmission of transgenic material by insect vectors to other neighbouring plants. Different species of leafhoppers, psyllids and aphids are known as vectors of phytopathogenic viruses and bacteria (Gottwald et al., 1995; Weintraub and Jones, 2010). They could suck on the adventitious shoots and are able to migrate over long distances (Taylor, 1974; Horten and Lewis, 1996; Harrington and Taylor 1990). Thereby they may be also responsible for the transmission and the spread of GM material.

In abandoned vineyards and orchards a potential risk for the development of suckers and furthermore flowers and seeds originating from adventitious shoots exists, in so far as releasing of transgenic material in the environment could be possible. In Austria, legislation of the federal provinces (Austrian regional plant protection laws and regional regulations for plant protection in pomi- and viticulture) regulates appropriate cultivation measures for vineyards and orchards, remedial measures, and the clearance of abandoned sites. In comparison, in commercial application the adventitious shoots get pruned down or a treatment with selected plant protection products is recommended.

6 Combination of techniques

It is generally conceivable that all new plant breeding techniques could be combined. However, some combinations appear more likely than others. Currently the different techniques are utilized to introduce specific traits in specific species. The trait/species-combination is mostly governed by the state of the art (*i.e.* possibilities to transform a given species or to induce a desired trait by a specific technique). As there is substantial progress concerning research and development in the new plant breeding techniques, new possibilities for combinations may arise.

Cisgenesis aims at introducing plant genes into the same or cross-compatible species, by definition without any changes to the sequences. However, it may be anticipated that the sequences may be altered after their insertion to change their expression levels. For this, **ZFN-1** or **ODM** techniques may be used. The cisgenes may be detected based on event-specific methods even when further modified. Small mutations introduced by ZFN or ODM may be detected but their origin may not be identified, as cisgenes may also be mutated naturally.

Intragenesis, in contrast to cisgenesis, allows for the combination of diverse genetic elements from the same or cross-compatible species, including silencing constructs; it may thus be used in combination with all approaches aiming at the silencing of endogenous genes, in particular **reverse breeding**.

If **cisgenesis** is combined with the **ZFN-3** technique the introduced cisgene will be inserted at a previously defined site. As sequences in cross-compatible species may be very similar, this fact may hinder the detection and unequivocal quantification of the introduced cisgene. In most cases, sufficient sequence diversity should be present to allow for detection. This question can only be answered after a case by case evaluation of the relevant sequences.

The **ZFN-3** technique can also be used in combination with **intragenesis**. Although this combination renders the insertion of the intragenic construct in a predefined place in the genome possible, detection is feasible due to the specific combination of sequences within the intragenic construct.

The **ZFN-1, -2** and **ODM** techniques may be used in combination with **agroinfiltration**; in this case, agroinfiltration serves as the method to introduce the molecules that result in the desired modifications in the genome. They are supposed to act only transiently, as is agroinfiltration. The changes introduced by the ZFN and ODM techniques will persist and may be detected, but not related to a specific technique or distinguished from naturally occurring mutations.

Agroinfiltration can be used to introduce the effector molecules into plants, in which genes should be silenced by **RdDM**. In contrast to RdDM induced by stable transformation of a plant, this combination is of particular interest related to the application of new plant breeding techniques as no modification to the plant genome *per se* occurs. This combination will not be detectable by standard methods.

Reverse breeding describes a procedure which is constituted of different techniques that are subsequently applied to reach the desired outcome – the reconstitution of completely homozygous parental lines. The major task is to suppress meiotic recombination. This can be reached by different approaches (RNA or DNA oligonucleotides, promoter silencing, VIGS, mutations to a target gene, chemically). To date the proof of concept has been shown by stable transformation with an RNAi construct. Similar effects could also be achieved by other dominantly acting gene silencing methods. All investigated methods that may potentially lead to gene silencing (**ZFN**, **ODM**, **RdDM**) might be suitable, as may be cisgenes or intragenes that could interact with the target gene by perturbing its function. However, currently there is no reason to anticipate that one of the new plant breeding methods would be the method of choice for reverse breeding in the near

future. The conclusions concerning detection would remain the same as discussed for the specific techniques. Finally, it will not be possible to retrace the application of reverse breeding in the final product. The safety issues related to the use of negative segregants would not be relevant if the meiotic recombination gene was silenced by transient techniques.

Grafting (on GM rootstock) is currently combined with standard GM techniques. Any new technique that allows producing a desired effect in a scion would be possible to use in combination but will be a matter of case-by-case proof of concept. Grafting has been mentioned as a suitable method to suppress meiotic recombination for reverse breeding.

7 Conclusions and recommendations

7.1 RdDM

Plants which have been altered by means of RdDM-technique can show different patterns in DNA-methylation of promoters, which results in expression or suppression of a gene or, at another level, in histone modification. Genes with strongly methylated promoters are generally inactive and, *vice versa*, promoters which are not methylated lead to unhampered expression of gene products.

The most important issues relevant for risk assessment are (for details please refer to chapter 3)

1. Plant-derived small RNAs are mobile
2. Spreading of the methylation cannot be predicted
3. Distribution of the silencing signal to non-target tissue
4. Gene silencing is not tissue-specific
5. The phenotypic stability of the silencing signal is unclear (amplification/fading of the signal is possible)
6. Variation in silencing effects seem to depend on the generation number
7. To date it has not been shown that RdDM has transgenerational stability
8. Promoter DNA methylation does not necessarily induce transcriptional inactivation
9. RNA silencing is environment dependent
10. Uncertainties concerning the fate and effects of ingested RNA molecules
11. The method is in the face of intense development
12. Scarce database

Based on current EFSA Guidance (EFSA 2011a), the following elements of the risk assessments of GM plants and derived food and feed should be considered:

1. Characteristics of the recipient plant
2. Genetic modification and its functional consequences
    - Description of the methods used for the genetic modification
    - Source and characterisation of nucleic acid used for transformation
    - *In silico* analysis has to be performed to assess potential unintended effects of the involved RNA sequences
    - In case of stable transformation: Nature and source of vector(s) used including nucleotide sequences intended for insertion. Absence of superfluous DNA sequences in the final product must be demonstrated.
    - Information on the sequences actually inserted/altered
    - Information on the expression of the inserted/modified sequence (*in vitro* and *in vivo*) including the flanking regions to characterise potential spreading of DNA methylation beyond the targeted nucleotide sequence
    - Characterisation of the alteration in and stability of the methylation pattern of the targeted DNA (regulatory) region
3. Agronomic and phenotypic characteristics of the plant, including compositional characteristics; general description of the trait(s) and characteristics which have been introduced or modified
    - Phenotypic stability of the plant
    - Equivalence tests/comprehensive comparative analyses between crops produced by RdDM and conventional counterparts

4. Potential toxicity and allergenicity of gene products and the whole plant and its derived products
    - pathophysiological role in humans or animals by influencing the expression of endogenous genes
    - *in vivo* animal studies that identify potential unforeseen consequences
5. Dietary impact and potential for nutritional impact (if applicable; case by case decision)
6. Environmental risk assessment
    - Analysis of potential alterations related to persistence and invasiveness; persistence of small RNA molecules
    - Impacts of specific cultivation, management and harvesting techniques
    - Higher susceptibility to plant diseases
    - Interaction of the GM plant with target organisms or non-target organisms; effects on soil microbes or related viruses and nematodes; non-target effects on organisms ingesting plants
    - Effects on biogeochemical processes
    - Effects on human and animal health
    - Unintended effects on molecular and cellular interactions

Detection

There are currently three main approaches to distinguish between 5mC and unmethylated cytosine in DNA:

- bisulfite conversion
- restriction enzymes
- affinity enrichment

The following preconditions are necessary to render detection of targeted methylation possible:

- Information on the intended alteration in the methylation pattern
- Map of the new methylation pattern
- Database for comparative analysis of methylation patterns in conventional plants and RdDM derived plants

The following restrictions concerning detection have been identified:

- RdDM induced methylation patterns cannot be distinguished from naturally occurring ones.
- Further research e.g. in relation to population genetics is necessary to gain insight into induced and naturally occurring variation of methylation.
- Generally, methylation patterns may also be changed through traditional plant breeding.

Conclusions

Core elements of the current risk assessment requirements for GM-plants are mandatory.

Unintended effects cannot be excluded, thus methods and procedures relating to the regulatory framework of GMOs should be considered.

Actual knowledge does not allow to differentiate between an RdDM induced methylation pattern and those not induced by RdDM, and therefore the plant cannot be unequivocally identified as a plant modified by RdDM by analytical means currently available.

Currently existing labelling regimes according to Regulation (EC) No 1829/2003 are not applicable, as unequivocal detection of the modification is not possible. Although methylation patterns may be analysed, their origin cannot be determined. To ensure traceability of plants and products derived from the use of RdDM an amendment of the regulative measures has to be considered. One possibility would be to declare the use of

the technique during the application for variety registration. Plants and products may then be traced by applying the standard measures relevant for the traceability for food, feed and seed.

Further research concerning the application of RdDM is necessary.

7.2 Reverse Breeding

To date, only one report is available, which describes the transformation of *A. thaliana* by the floral dip method. The following assessment is based on this publication (Wijnker et al. 2012).

- All potential risks associated with *Agrobacterium*-mediated transformation need to be considered.
- Using RNAi the unintended effects due to short RNAs have to be excluded by *in silico* analyses.
- Reverse breeding includes the production of doubled haploids which being an *in vitro* method is prone to somaclonal variation; however, DH is state of the art in modern traditional plant breeding. In addition, plant breeders apply rigorous selection during the breeding process. The variety registration includes testing of Value for Cultivation and Use (VCU) over several years and in different environments. These procedures should widely minimize risks associated with the traditional breeding process.

Negative segregants constitute the final product of reverse breeding. They are not to be used as comparators as they lack history of safe use and unintended effects due to genetic modification could have occurred (EFSA 2011b). In this sense, the same applies to the negative segregants resulting from the reverse breeding process.

The intermediate product of reverse breeding (the GM plant) should be thoroughly risk assessed concerning its molecular characteristics based on current EFSA Guidance (EFSA 2011a). In particular, this includes

- Description of the methods used for the genetic modification.
- Source and characterization of nucleic acid used for transformation.
- Nature and source of vector(s) used including nucleotide sequences intended for insertion.
- Information on the sequences actually inserted/deleted or altered. The copy number of intact and fragmented inserts should be established.
- Information on the expression of the inserted/modified sequence including the flanking regions to exclude unintended effects related to the transformation process.

Concerning environmental risk assessment it is important to perform *in silico* analyses (in particular gene homologies) to largely exclude potential interactions with other organisms.

As the negative segregant potentially contains unintended modifications due to the transformation process, their presence should be largely excluded by the analysis of the stably transformed plant. The negative segregant itself should not contain the insert; its absence has to be verified.

In conclusion the negative segregant at some stage needs to be treated similar to a GMO plant.

Detection

Detection is possible in the stably transformed plant. As the insert is not present in the final product (negative segregant), it is not possible to retrace the origin of the plant.

Conclusions

According to the state of the art negative segregants should be treated like GMOs. Detection in the final product is not possible, traceability can only be ensured by continuous documentation. GMOs are labelled by footnotes in the Common catalogues of varieties (currently this applies to varieties of maize and potato, which have been authorised for cultivation in the European Union). The products of reverse breeding could be labelled accordingly. However, currently existing labelling regimes according to Regulation (EC) No 1829/2003

are not applicable. The use of the technique can neither be detected in the final product nor is quantification possible as commercialised plants are derived from negative segregants that by definition should not contain traces of the transgene. Traceability based on continuous documentation would be possible. In this context, the regulatory framework has to be discussed; provisions for the declaration of the use of the technique during the variety registration process would have to be adopted.

7.3 Grafting

Grafting *per se* is a method which is state of the art for breeding of grapevine and fruit trees. In this context, grafting has a history of safe use. New risks may be associated with this breeding technique if GMOs are involved. The following potential risks may arise from grafting of a non-GM scion onto a GM rootstock:

- Transgenic metabolites (proteins, hormones, siRNAs, etc.) can be transported from the transgenic rootstock to the upper stem where they accumulate and cause an effect.
- These mobile metabolites may have unanticipated effects on, e.g., phytoplasms, nematodes, psyllids, aphids, including their potential transfer.
- There is currently no sufficient database concerning potential effects on humans and animals consuming products of these plants.
- Adventitious shoots may be a source of unintended effects, in particular if they emerge from the transgenic rootstock.

When produced through standard transformation methods, the risk assessment of the GM rootstock should be fully performed according to the current EFSA Guidance (EFSA 2011a). This should include the analysis of the products that are to be consumed by humans and/or animals. Special attention should be paid to the environmental risk assessment (EFSA 2010), in particular for perennials like grapevine or trees.

Substantial parts of the grafted plant are GM; the thorough risk assessment of the GM part should help to exclude the risks reflected in the EFSA guidance. For the transgrafted plant an *in silico* assessment taking into consideration the characteristics of the two different genotypes and potential interactions should suffice. Special action is required when potential risks (e.g. concerning toxicological effects) have been identified on a case-by-case basis.

Detection

The transgenic nature of the rootstock – when produced by currently used methods for genetic modification – can be detected unequivocally following standard procedures.

Molecules (e.g. proteins) produced by the GM rootstock may be readily detectable given that information on the modification and the intended effect is available; however, it has to be determined on a case-by-case basis whether it is possible to distinguish them from naturally occurring molecules. Depending on the GM mechanism, no detectable molecules may be found in the plant products.

In conclusion, a case-by-case evaluation has to be performed. No general recommendation concerning the potential to identify and distinguish molecules derived from the transgenic rootstock is possible. Major limitations may be expected related to the possibilities for quantification.

Conclusions

If the rootstock is transformed by standard GM techniques all current risk assessment procedures apply. Detection and quantification is possible in this case. Further research concerning the potential risks associated with the movement of molecules *in planta* is necessary, as there is no conclusive information available as to the risks emerging from particular transgenic metabolites. Due to the great variation in potential effects of the

molecules produced by the transgene that may affect the whole plant and derived products a case-by-case evaluation will be necessary. On the same basis, effects on the environment have to be assessed, and possibilities for identification and quantification will have to be evaluated. Generally, the regulatory framework concerning the relevant plants and their products should be reconsidered.

8 Annex

8.1 RdDM – Scientific peer-reviewed literature reporting experimental data

Chalfun-Junior A, Mes JJ, Mlynarova L, Aarts MGM, Angenent GC (2003). Low frequency of T-DNA based activation tagging in *Arabidopsis* is correlated with methylation of CaMV 35S enhancer sequences.

Abstract
A powerful system to create gain-of-function mutants in plants is activation tagging using T-DNA based vehicles to introduce transcriptional enhancer sequences. Large *Arabidopsis* populations of individual plants carrying a quadruple cauliflower mosaic virus (CaMV) 35S enhancer are frequently used for mutant screenings, however the frequency of morphological mutants remains very low. To clarify this low frequency we analyzed a subset of lines generated by this method. The correlation between the number of T-DNA insertion sites, the methylation status of the 35S enhancer sequence and 35S enhancer activity was determined. All plants containing more than a single T-DNA insertion showed methylation of the 35S enhancer and revealed a dramatic decrease in 35S enhancer activity. The results support the notion that in a large proportion of the T-DNA based activation tagged lines the 35S transcriptional enhancer is silenced due to methylation, which is induced by multiple T-DNA integrations.

Chan SW, Zhang X, Bernatavichute YV, Jacobsen SE (2006). Two-step recruitment of RNA-directed DNA methylation to tandem repeats.

Abstract
Tandem repeat sequences are frequently associated with gene silencing phenomena. The *Arabidopsis thaliana* FWA gene contains two tandem repeats and is an efficient target for RNA-directed de novo DNA methylation when it is transformed into plants. We showed that the FWA tandem repeats are necessary and sufficient for de novo DNA methylation and that repeated character rather than intrinsic sequence is likely important. Endogenous FWA can adopt either of two stable epigenetic states: methylated and silenced or unmethylated and active. Surprisingly, we found small interfering RNAs (siRNAs) associated with FWA in both states. Despite this, only the methylated form of endogenous FWA could recruit further RNA-directed DNA methylation or cause efficient de novo methylation of transgenic FWA. This suggests that RNA-directed DNA methylation occurs in two steps: first, the initial recruitment of the siRNA-producing machinery, and second, siRNA-directed DNA methylation either in *cis* or in *trans*. The efficiency of this second step varies depending on the nature of the siRNA-producing locus, and at some loci, it may require pre-existing chromatin modifications such as DNA methylation itself. Enhancement of RNA-directed DNA methylation by pre-existing DNA methylation could create a self-reinforcing system to enhance the stability of silencing. Tandem repeats throughout the *Arabidopsis* genome produce siRNAs, suggesting that repeat acquisition may be a general mechanism for the evolution of gene silencing.

Dalakouras A, Moser M, Zwiebel M, Krczal G, Hell R, Wassenegger M (2009). A hairpin RNA construct residing in an intron efficiently triggered RNA-directed DNA methylation in tobacco.

Abstract
So far, conventional hairpin RNA (hpRNA) constructs consisting of an inverted repeat (IR) of target promoters directly introduced into an expression cassette have been used to mediate de novo DNA methylation. Transcripts of such constructs resemble mRNA molecules, and are likely to be exported to the cytoplasm. The presence of hpRNAs in the cytoplasm and the nucleus may account for the simultaneous activation of post-transcriptional gene silencing (PTGS) and RNA-directed DNA methylation (RdDM). We hypothesized that by

retaining hpRNAs in the nucleus, efficient induction of only RdDM may be achieved. Thus, we introduced into tobacco a transgene containing an intron into which an IR of a target promoter was inserted. The intronic hpRNA initiated highly specific *cis*- and *trans*-methylation, but did not induce PTGS. No spreading of methylation into sequences flanking the region of homology between the hpRNA and the target DNA was detectable. The efficient methylation-directing activity of the intronic hpRNA may indicate a previously unrecognized role of introns, potentially regulating gene expression at the transcriptional level.

Dalakouras A, Tzanopoulou M, Tsagris M, Wassenegger M, Kalantidis K (2011). Hairpin transcription does not necessarily lead to efficient triggering of the RNAi pathway.

Abstract
Previously, we had shown that stable expression of a hairpin RNA sharing homology with the coat protein (CP) of the Cucumber mosaic virus (CMV) (hpRNA(CMV)) produced CMV resistant *Nicotiana tabacum* plants. However, only 17% of the hpRNA(CMV)-expressing plants generated substantial amounts of siRNAs that mediated CMV resistance (siRNAs(CMV)). Here, we demonstrate that the transcription of a hpRNA(CMV) per se is not sufficient to trigger cytoplasmic and nuclear RNAi. A multiple-transgene copy line showed a strong resistance phenotype. Segregation of individual copies revealed that in one locus, the transgene-produced hpRNA(CMV) transcript was processed into 21-nt and 24-nt siRNAs(CMV) and lines containing this locus were resistant. At a second locus, where the transgene was shown to be transcribed, no siRNAs(CMV) were produced and lines harbouring only this locus were susceptible. In addition, the second locus failed to trigger *de novo* RNA-directed DNA methylation (RdDM) in cis, of its cognate sequence. However, after being induced in *trans*, methylation in the transcribed region of the transgene was maintained in both CG and CHG residues. Sequence-specific maintenance of methylation in transcribed regions, as well as diverse RNA degradation pathways in plants are discussed in view of our observations.

Febres VJ, Lee RF, Moore GA (2008). Transgenic resistance to Citrus tristeza virus in grapefruit.

Abstract
Grapefruit (*Citrus paradisi*) transgenic plants transformed with a variety of constructs derived from the Citrus tristeza virus (CTV) genome were tested for their resistance to the virus. Most transgenic lines were susceptible (27 lines), a few were partially resistant (6 lines) and only one line, transformed with the 3' end of CTV was resistant. Transgene expression levels and siRNA accumulation were determined to identify whether the resistance observed was RNA-mediated. The responses were varied. At least one resistant plant from a partially resistant line showed no steady-state transgene mRNA, siRNA accumulation and no viral RNA, implicating posttranscriptional gene silencing (PTGS) as the mechanism of resistance. The most resistant line showed no transgene mRNA accumulation and promoter methylation of cytosines in all contexts, the hallmark of RNA-directed DNA methylation and transcriptional gene silencing (TGS). The variety of responses, even among clonally propagated plants, is unexplained but is not unique to citrus. The genetics of CTV, host response or other factors may be responsible for this variability.

Fischer U, Kuhlmann M, Pecinka A, Schmidt R, Mette MF (2008). Local DNA features affect RNA-directed transcriptional gene silencing and DNA methylation.

Abstract
Transcription of a nopaline synthase promoter (pNOS) inverted repeat provides an RNA signal that can trigger transcriptional gene silencing and methylation of pNOS promoters *in trans*. The degree of silencing is influenced by the local DNA features close to the target promoter integration sites. Among 26 transgenic *Arabidopsis thaliana* lines harbouring single copies of a T-DNA including a pNOS-*NPTII* reporter gene at different chromosomal loci, *NPTII* RNA levels showed limited variation. When challenged by the silencer

transgene providing the pNOS RNA signal, reduction of the *NPTII* RNA levels in the F(1) generation varied by more than 100-fold, ranging from no reduction to reduction to < 1% of the non-silenced level. Silencing was generally correlated with proportional DNA methylation in the pNOS region, except for one target transgene showing substantial DNA methylation without adequate silencing. Silencing was progressive through generations. Differences in the degree of silencing among the target transgenes were transmitted at least to the F(3) generation, and seemed to be influenced by transgene-flanking sequences. Apparently, close-by repeats promoted, whereas close-by functional genes diminished, the response to the silencing signal.

Kanazawa A, Inaba J, Shimura H, Otagaki S, Tsukahara S, Matsuzawa A, Kim BM, Goto K, Masuta C (2011). Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants.

Abstract

Gene silencing through transcriptional repression can be induced by targeting double-stranded RNA (dsRNA) to a gene promoter. It has been reported that a transgene was silenced by targeting dsRNA to the promoter, and the silenced state was inherited to the progeny plant even after removal of the silencing inducer from cells. In contrast, no plant has been produced that harbors silenced endogenous gene after removal of promoter-targeting dsRNA. Here, we show that heritable gene silencing can be induced by targeting dsRNA to the endogenous gene promoters in petunia and tomato plants, using the Cucumber mosaic virus (CMV)-based vector. We found that efficient silencing of endogenous genes depends on the function of the 2b protein encoded in the vector virus, which has the ability to facilitate epigenetic modifications through the transport of short interfering RNA to nucleus. Bisulfite sequencing analyses on the targeted promoter in the virus-infected and its progeny plants revealed that cytosine methylation was found not only at CG or CNG but also at CNN sites. The observed inheritance of asymmetric DNA methylation is quite unique, suggesting that plants have a mechanism to maintain even asymmetric methylation. This CMV-based gene silencing system provides a useful tool to artificially modify DNA methylation in plant genomes and elucidate the mechanism for epigenetic controls.

Miki D, Shimamoto K (2008). *De novo* DNA methylation induced by siRNA targeted to endogenous transcribed sequences is gene-specific and *OsMet1*-independent in rice.

Abstract

Small interfering RNA (siRNA) is an essential factor for epigenetic modification of the genome. Recent studies have suggested that endogenous siRNAs induce DNA methylation, chromatin modification and chromatin inactivation at homologous sequences. We have shown that siRNAs targeted to promoter regions of endogenous rice genes induce strong DNA methylation of the targeted sequences, but transcriptional gene silencing is rarely observed. Here, an analysis of epigenetic modifications induced by RNAi targeted to transcribed regions of endogenous rice genes shows that the effects of siRNA are gene-specific, but that they tend to induce higher *de novo* DNA methylation of CpG dinucleotides than of other cytosines. However, loss of *OsMet1* expression by RNAi did not significantly affect levels and patterns of *de novo* DNA methylation or post-transcriptional mRNA suppression. We also showed that sequence-specific *de novo* DNA methylation extended both 5' and 3' of the targeted sequences, but there was no significant extension of siRNA signals either 5' or 3'. These results suggest that exogenous siRNAs are strong inducers of *de novo* DNA methylation in transcribed sequences of rice endogenous genes, but are insufficient to induce heterochromatin formation.

Mourrain P, van Blokland R, Kooter JM, Vaucheret H (2007). A single transgene locus triggers both transcriptional and post-transcriptional silencing through double-stranded RNA production.

Abstract
Silencing of a target locus by an unlinked silencing locus can result from transcription inhibition (transcriptional gene silencing; TGS) or mRNA degradation (post-transcriptional gene silencing; PTGS), owing to the production of double-stranded RNA (dsRNA) corresponding to promoter or transcribed sequences, respectively. The involvement of distinct cellular components in each process suggests that dsRNA-induced TGS and PTGS likely result from the diversification of an ancient common mechanism. However, a strict comparison of TGS and PTGS has been difficult to achieve because it generally relies on the analysis of distinct silencing loci. We describe a single transgene locus that triggers both TGS and PTGS, owing to the production of dsRNA corresponding to promoter and transcribed sequences of different target genes. We describe mutants and epigenetic variants derived from this locus and propose a model for the production of dsRNA. Also, we show that PTGS, but not TGS, is graft-transmissible, which together with the sensitivity of PTGS, but not TGS, to RNA viruses that replicate in the cytoplasm, suggest that the nuclear compartmentalization of TGS is responsible for cell-autonomy. In contrast, we contribute local and systemic trafficking of silencing signals and sensitivity to viruses to the cytoplasmic steps of PTGS and to amplification steps that require high levels of target mRNAs.

Okano Y, Miki D, Shimamoto K (2008). Small interfering RNA (siRNA) targeting of endogenous promoters induces DNA methylation, but not necessarily gene silencing, in rice.

Abstract
Recent evidence indicates that small interfering RNA (siRNA) induces chromatin modifications and inactivation at homologous genomic sequences. A large number of endogenous siRNAs have been discovered that correspond to widely dispersed regions of the genome. We used an experimental system in which transgene-derived siRNAs target promoter regions in rice to determine whether or not siRNAs induce chromatin modifications that result in inactivation. Our results indicate that siRNAs targeted to one transgene and seven endogenous genes induce DNA methylation at all of the target promoters, but do not induce transcriptional suppression. Chromatin immunoprecipitation (ChIP) assays indicate that reduced euchromatic histone modifications were concomitant with the silencing of one endogenous gene, but not of six other endogenous genes that were not silenced. Furthermore, heterchromatic H3K9me2 was higher only in the promoter of the transgene that was completely silenced. These findings lead us to assume that siRNA rarely induces chromatin inactivation or changes in pattern of histone modification, especially H3K9 methylation, within most regions of the genome.

Shibuya K, Fukushima S, Takatsuji H (2009). RNA-directed DNA methylation induces transcriptional activation in plants.

Abstract
A class-C floral homeotic gene of *Petunia*, *pMADS3*, is specifically expressed in the stamen and carpels of developing flowers. We had previously reported the ect-*pMADS3* phenomenon in which introduction of a part of the *pMADS3* genomic sequence, including intron 2, induces ectopic expression of endogenous *pMADS3*. Unlike transcriptional or posttranscriptional gene silencing triggered by the introduction of homologous sequences, this observation is unique in that the gene expression is up-regulated. In this study, we demonstrated that the ect-*pMADS3* phenomenon is due to transcriptional activation based on RNA-directed DNA methylation (RdDM) occurring in a particular CG in a putative *cis*-element in *pMADS3* intron 2. The CG methylation was maintained over generations, along with *pMADS3* ectopic expression, even in the absence of RNA triggers. These results demonstrate a previously undescribed transcriptional regulatory mechanism that could lead to the generation of a transcriptionally active epiallele, thereby contributing to plant evolution. Our

results also reveal a putative negative *cis*-element for organ-specific transcriptional regulation of class-C floral homeotic genes, which could be difficult to identify by other approaches.

8.2 Reverse Breeding - Scientific peer-reviewed literature reporting experimental data

Wijnker et al. (2012) Reverse breeding in *Arabidopsis thaliana* generates homozygous parental lines from a heterozygous plant.

Abstract

Traditionally, hybrid seeds are produced by crossing selected inbred lines. Here we provide a proof of concept for reverse breeding, a new approach that simplifies meiosis such that homozygous parental lines can be generated from a vigorous hybrid individual. We silenced DMC1, which encodes the meiotic recombination protein DISRUPTED MEIOTIC cDNA1, in hybrids of *A. thaliana*, so that non-recombined parental chromosomes segregate during meiosis. We then converted the resulting gametes into adult haploid plants, and subsequently into homozygous diploids, so that each contained half the genome of the original hybrid. From 36 homozygous lines, we selected 3 (out of 6) complementing parental pairs that allowed us to recreate the original hybrid by intercrossing. In addition, this approach resulted in a complete set of chromosome-substitution lines. Our method allows the selection of a single choice offspring from a segregating population and preservation of its heterozygous genotype by generating homozygous founder lines.

8.3 Grafting – Scientific peer-reviewed literature reporting experimental data

Agüero,C. B., S. L. Uratsu, C. Greve, A. L. T. Powell, J. M. Labavitch, C. P. Meredith, A. M. Dandekar, 2005, Evaluation of tolerance to Pierce's disease and Botrytis in transgenic plants of *Vitis vinifera L.* expressing the pear PGIP gene:

Polygalacturonase-inhibiting proteins (PGIPs) are plant cell-wall proteins that specifically inhibit fungal endo-polygalacturonases (PGs) that contribute to the aggressive decomposition of susceptible plant tissues. The inhibition of fungal PGs by PGIPs suggests that PGIPs have a role in plant tolerance to fungal infections and this has been observed in transgenic plants expressing PGIPs. *Xylella fastidiosa*, the causal agent of Pierce's disease (PD) in grapevines, has genes that encode cell-wall-degrading enzymes, including a putative PG. Therefore, we hypothesized that PGIP expression could confer tolerance against this bacterium as well as against the fungal pathogen Botrytis cinerea. To test this hypothesis, *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' were transformed to express pear fruit PGIP-encoding gene (pPGIP) under the control of the CaMV 35S promoter. Substantial pear PGIP (pPGIP) activity was found in crude extracts from leaves and in xylem exudate of transgenic lines obtained from independent transformation events, but not in untransformed controls. pPGIP activity was detected in xylem exudate of untransformed scions grafted on to transgenic rootstocks expressing pPGIP. Leaves of transgenic plants infected with *B. cinerea* had reduced rates of lesion expansion. The development of PD was delayed in some transgenic lines with increased pPGIP activity. PD-tolerant transgenic lines had reduced leaf scorching, lower *Xylella* titres and better re-growth after pruning than the untransformed controls.

Jensen, P. J, N. Halbrendt, G. Fazio, I. Makalowska, N. Altman, C. Praul, S. N. Maximova, H. K. Ngugi, R. M. Crassweller, J. W. Travis and T. W. McNellis, 2012, Rootstock-regulated gene expression patterns associated with fire blight resistance in apple

Abstract

Background

Desirable apple varieties are clonally propagated by grafting vegetative scions onto rootstocks. Rootstocks influence many phenotypic traits of the scion, including resistance to pathogens such as *Erwinia amylovora*, which causes fire blight, the most serious bacterial disease of apple. The purpose of the present study was to quantify rootstock-mediated differences in scion fire blight susceptibility and to identify transcripts in the scion whose expression levels correlated with this response.

Results

Rootstock influence on scion fire blight resistance was quantified by inoculating three-year old, orchard-grown apple trees, consisting of 'Gala' scions grafted to a range of rootstocks, with *E. amylovora*. Disease severity was measured by the extent of shoot necrosis over time. 'Gala' scions grafted to G.30 or MM.111 rootstocks showed the lowest rates of necrosis, while 'Gala' on M.27 and B.9 showed the highest rates of necrosis. 'Gala' scions on M.7, S.4 or M.9F56 had intermediate necrosis rates. Using an apple DNA microarray representing 55,230 unique transcripts, gene expression patterns were compared in healthy, un-inoculated, greenhouse-grown 'Gala' scions on the same series of rootstocks. We identified 690 transcripts whose steady-state expression levels correlated with the degree of fire blight susceptibility of the scion/rootstock combinations. Transcripts known to be differentially expressed during *E. amylovora* infection were disproportionately represented among these transcripts. A second-generation apple microarray representing 26,000 transcripts was developed and was used to test these correlations in an orchard-grown population of trees segregating for fire blight resistance. Of the 690 transcripts originally identified using the first-generation array, 39 had expression levels that correlated with fire blight resistance in the breeding population.

Conclusions

Rootstocks had significant effects on the fire blight susceptibility of 'Gala' scions, and rootstock-regulated gene expression patterns could be correlated with differences in susceptibility. The results suggest a relationship between rootstock-regulated fire blight susceptibility and sorbitol dehydrogenase, phenylpropanoid metabolism, protein processing in the endoplasmic reticulum, and endocytosis, among others. This study illustrates the utility of our rootstock-regulated gene expression data sets for candidate trait-associated gene data mining.

Gal-On, A., D. Wolf, Y. Antignus, L. Patlis, Ki Hyun Ryu, B. Eun Min, M. Pearlsman, O. Lachman, V. Gaba,and Y. Wang, 2005, Transgenic cucumbers harboring the 54-kDa putative gene of *Cucumber fruit mottle mosaic tobamovirus* are highly resistant to viral infection and protect non-transgenic scions from soil infection:

Cucumber fruit mottle mosaic tobamovirus (CFMMV) causes severe mosaic symptoms and yellow mottling on leaves and fruits and, occasionally, severe wilting of cucumber (*Cucumis sativus* L.) plants. No genetic source of resistance against this virus has been identified in cucumber. The gene coding for the putative 54-kDa replicase gene of CFMMV was cloned into an *Agrobacterium tumefaciens* binary vector, and transformation was performed on cotyledon explants of a parthenocarpic cucumber cultivar. R1 seedlings were screened for resistance to CFMMV by symptom expression, back inoculation on an alternative host and ELISA. From a total of 14 replicase-containing R1 lines, eight resistant lines were identified. Line I44 – homozygous for the putative 54-kDa replicase gene – was immune to CFMMV infection by mechanical and graft inoculation, and to root

infection following planting in CFMMV-infested soil. A substantial delay of symptom appearance was observed following infection by three additional cucurbit-infecting tobamoviruses. When used as a rootstock, line I44 protected susceptible cucumber scions from soil infection by CFMMV. This paper is the first report on protection of a susceptible cultivar against a soil-borne viral pathogen, by grafting onto a transgenic rootstock.

Ruiz, J.M. , J. J. Ríos, M. A. Rosales, R. M. Rivero, L. Romero, 2006, Grafting between tobacco plants to enhance salinity tolerance:

We analysed the technique of grafting as a tool to increase salt-stress resistance in tobacco plants. With this aim, we performed two experiments. First, we selected, from among 6 commercial tobacco cultivars (cv. BB-162, cv. H-20, cv. Jarandilla, cv. ZB-3, cv. Havana II and cv. Havana 307) those most tolerant and sensitive to salinity, studying the response of certain nutritional and biochemical indicators of resistance in these plants. In the second experiment, we analysed the response to salinity in grafted tobacco plants using the rootstock of the most tolerant plants, and the scion of the most sensitive ones. In addition, these plants were subjected to salinity to test the viability and efficiency of this grafting technique, assessing the production of foliar biomass and the different quality parameters in this crop. In the first experiment, we found that the most tolerant tobacco cultivars were cv. BB-162 and cv. H-20, which were characterized by reduced uptake and foliar accumulation of Na+ and Cl−, together with greater synthesis of sucrose and proline, thereby reducing lipid peroxidation and thus oxidative damage, reflected in higher foliar biomass with respect to the other cultivars studied (primarily cv. Jarandilla, defined as the most salt-sensitive). In the second, we demonstrated that the grafting of salt-sensitive tobacco scions to salt-tolerant rootstocks improves the production and quality of tobacco leaves under conditions of saline stress. Our results show that the rootstocks cv. BB-162 and cv. H-20 best induced salt resistance in tobacco cv. Jarandilla, registering the lowest foliar concentrations of Na+ and Cl+, the lowest lipid peroxidation, and the highest proline and sugar concentrations. Overall, this is reflected in better biomass production and quality of the aerial part of the plant.

Song, G., Kenneth C. Sink, Aaron E. Walworth, Meridith A. Cook, Richard F. Allison, Gregory A. Lang, 2013, Engineering cherry rootstocks with resistance to Prunus necrotic ring spot virus through RNAi-mediated silencing:

Prunus necrotic ringspot virus (PNRSV) is a major pollen-disseminated ilarvirus that adversely affects many *Prunus* species. In this study, an RNA interference (RNAi) vector pART27–PNRSV containing an inverted repeat (IR) region of PNRSV was transformed into two hybrid (triploid) cherry rootstocks, ‘Gisela 6’ (GI 148-1) and ‘Gisela 7’(GI 148-8)’, which are tolerant and sensitive, respectively, to PNRSV infection. One year after inoculation with PNRSV plus Prune Dwarf Virus, nontransgenic ‘Gisela 6’ exhibited no symptoms but a significant PNRSV titre, while the transgenic ‘Gisela 6’ had no symptoms and minimal PNRSV titre. The nontransgenic ‘Gisela 7’ trees died, while the transgenic ‘Gisela 7’ trees survived. These results demonstrate the RNAi strategy is useful for developing viral resistance in fruit rootstocks, and such transgenic rootstocks may have potential to enhance production of standard, nongenetically modified fruit varieties while avoiding concerns about transgene flow and exogenous protein production that are inherent for transformed fruiting genotypes.

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