

New plant breeding Techniques

Risks Associated with their Application



NEW PLANT BREEDING TECHNIQUES AND RISKS ASSOCIATED WITH THEIR APPLICATION

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1 INTRODUCTION

1.1 Background and aim of the study

This report aims to provide an overview and a discussion of biosafety-related aspects of the application of biotechnology-based approaches in plant breeding, other than genetically modified (GM) crops as defined in the European regulations on genetically modified organisms (GMOs), specifically the respective regulations existing in the EU and its Member States (e.g. Directive 2001/18/EC) and the Swiss Federation (e.g. Federal Gene Technology Act SR 814.91, Release Ordinance; SR 814.911). Such applications, which are also called „new plant breeding techniques“ (NPBT), are different to both conventional breeding approaches as well as standard GM developments (LUSSEY et al. 2012).

Whereas biosafety issues of the application of GM crops were actively and controversially discussed in the last decades, the application of other biotechnological approaches only received more attention in recent years. This is mostly due to the fact that the existing biosafety frameworks mandate that a risk assessment is conducted for GM crops ahead of authorisation of use, e.g. for import and production of food and feeds as well as for cultivation in Europe. Thus a mandatory risk assessment needs to be provided for all such GM crops since introduction of the respective regulations 1990 in the European Union.

Other biotechnology-based applications with relevance for crop breeding were not similarly in the focus of public attention and debate. Only in the recent years a debate started which is mostly focused on the issue whether such applications would be subject to regulation according to the existing biosafety laws (cf. PODEVIN et al. 2012).

This debate at present is fuelled by an increase in activities of seed developing companies to use such NPBT for commercial developments, taking into account that some of these NPBTs may offer a number of advantages compared with GM technology. On the one hand such approaches could be technologically favorable, i.e. offer more appropriate solutions for specific breeding objectives than common GM-developments. On the other hand NPBTs may be exempt from GMO regulation and thus provide a faster road to commercialization of products. While some of these technologies – contrary to the denomination as “new” – were developed some decades ago, they are regarded to be more viable approaches of developments only in the recent years due to technological advances and broader access to these technologies by seed developing institutions.

Independent from any legal aspects it is evident that NPBTs may be used to introduce “new” traits into crop varieties, e.g. traits directed to enhance resistance against different types of pathogens, to introduce tolerance against broad spectrum herbicides, as well as to modify the substantial composition of certain crops or other phenotypic and reproduction characteristics (cf. WALTZ 2012).

This study therefore aims to provide a discussion of the potential adverse effects on human health and the environment which might be associated with an application of NPBTs in crop development. Wherever appropriate, the analysis also takes into consideration that quite often a combination of technologies (e.g. NPBT & conventional breeding, NPBT & GM technology, different NPBTs used in combination) is applied to achieve certain breeding objectives. Additionally

the study takes into account that some adverse effects are rather associated with the practical application of a crop variety produced by a NPBT, e.g. the agricultural management of such a crop during cultivation, than with the technology used for breeding or the crop variety itself.

The objective of the study is to present an overview on the scientific state of knowledge as regards the potential adverse effects of certain NPBTs and the agro-ecological impacts associated with the cultivation of NPBT-crops in present agricultural settings. The results of the study should provide a background for the review of the current biosafety requirements for NPBT-crops by advisory and regulatory bodies. Such a review may identify necessary revisions of the biosafety framework as regards potential effects associated with NPBT-crops in comparison with health related and environmental impacts of crops developed by conventional plant breeding and GM-crops.

To meet these aims the first part of this study is dedicated to an overview of specific NPBTs, indicating their potential combinations and their implication in breeding schemes. It further identifies characteristics of the resulting crops, which are regarded as relevant for the analysis of potential adverse effects of the application of such crops.

This review is based on the scientific literature concerning the respective NPBTs and their application available at present. In addition the biosafety considerations in this review build on previous studies addressing other aspects of NPBTs, specifically legal aspects whether NPBT-crops will be subject to the existing regulation frameworks for GM-crops. Such reviews have been conducted by EU institutions, e.g. by the Working Group on New Techniques (NTWG) of the European Commission (NTWG 2011), the Joint Research Center (JRC) of the European Commission (LUSSE et al. 2011) and EFSA (EFSA 2012 a & 2012b) as well as other (Member) State Institutions, e.g. AGES – Austrian Agency for Health and Food Safety (AGES 2012), or COGEM - Dutch Commission on Genetic Modification (COGEM 2006).

The second part of the study is dedicated to a qualitative assessment of the potential of NPBTs to result in intended and unintended (genetic) changes in the resultant crops as well as the potential impacts of practical applications of such crops. Adverse effects which need to be assessed for GM crops according to the biosafety regulation framework are specifically taken into consideration for this analysis (cf. EFSA 2010 & 2011). As appropriate the identified “risk issues” associated with NPBT-bred crops are compared with related impacts of GM crops with analogous traits or of conventionally bred crops.

The study is further analyzing whether the approaches and criteria developed for the assessment of GM crops are relevant and sufficient to address the potential risks associated with crops developed by application of NPBT or whether specific improvements of such criteria are necessary to adequately address NPBT-bred crops. For this analysis the study also takes into consideration suggestions for the further improvement of risk assessment for GM crops (DOLEZEL et al. 2011, HEINEMANN et al. 2013).

1.2 NPBTs addressed in this study

The following table lists the individual NPBTs selected for discussion in the present study (Tab. 1, for a detailed discussion of individual techniques see Chapter 2).

New plant breeding technique (NPBT)	Discussion in Chapter
Cell fusion techniques (Protoplast fusion)	2.1
Marker Assisted Selection (MAS)	2.2
Oligonucleotide-directed mutagenesis (ODM)	2.3
Nuclease-mediated site-directed mutagenesis Zinc Finger Nucleases (ZFN), Transcription Activator-like Effector Nuclease (TALEN), Meganuclease (MN), CRISPR-Cas-Nucleases	2.4
Cisgenesis & Intragenesis	2.5
Grafting on GM Rootstock (Transgrafting)	2.6
Techniques to support breeding Reverse breeding, Seed production technology, Accelerated breeding	2.7
Agroinfiltration	2.8

*Table 1:
Overview on NPBTs
discussed in the report
at hands:*

From an overall perspective these NPBTs are a group of techniques, which are very diverse as regards their approach, methodology and unique characteristics. They may either be used alone in the breeding process of a certain crop or they may be used in combination with other NPBTs, conventional breeding approaches or together with GM technology in breeding. While some NPBTs share certain commonalities as regards the underlying methodological approaches or the implicated molecular mechanisms, the differences between individual techniques encountered across the range of NPBTs covered in this report are substantial.

Therefore some previous reviewers which tried to address the full range of NPBTs did not try to further categorise the different NPBTs into a single comprehensive classification system (see e.g. NTWG 2011, LUSSEK et al. 2011). However types of NPBTs which constitute modifications of one specific approach were grouped together (as above in Tab. 1) for a detailed discussion (e.g. different types of ZFN-applications and other techniques based on targeted (i.e. sequence-specific) nucleases to introduce genetic modifications, Cisgenesis & Intragenesis applications, different types of Agro-Infiltration approaches).

Some subsequent reviews (cf. LUSSEK et al. 2012, SCHAART & VISSER 2009; PODEVIN et al. 2012) did attempt to provide further categorisations of the NPBTs to streamline the evaluation of NPBTs. However the discussion in these reviews did not focus on the risk assessment of NPBTs, but rather on the question, whether such NPBTs are subject to existing regulation frameworks for biotechnology in the EU as well as in different countries (cf. LUSSEK et al. 2012). An overview on the different categorisation schemes and the criteria underlying the chosen systems is provided in Annex 1 to this report, since some of the criteria used for categorisation by LUSSEK et al. (2012) and PODEVIN et al. (2012) are also relevant for the assessment of certain potential risks, which may be associated with a specific application of NPBTs.

However from the viewpoint of risk assessment no single classification scheme is appropriately integrating all requirements necessary for a comprehensive approach to risk assessment. Since the different risk assessment requirements will probably not allow for development of a fully appropriate hierarchical categorization of NPBTs, the discussion presented in Chapter 2 of this report will address the selected NPBTs individually without introducing any additional classification system.

Nevertheless any risk assessment for individual NPBT-crops needs to be conducted in a structured and comparable manner. The discussion in Chapter 2 will therefore be based on the following set of considerations (see Tab. 2).

Table 2: Overview on the risk assessment considerations concerning NPBTs used in the report at hands:

Categories for consideration	Issues for Consideration
Intended modification by NPBT	1) Is a genetic modification introduced intentionally into the breeding product? 1.1) What kind of genetic modification is introduced? - Targeted mutation in a genetic element - Non-targeted mutation(s) in plant genome - Knock-out of native gene(s) - Introduction of modified gene(s) - gene “knock-In” 1.2) How stable are the introduced genetic modifications? 2) Are epigenetic modifications intentionally introduced in the breeding product? 2.1) What kind of epigenetic modification is introduced? - Which epigenetic mechanism is targeted? - What is the expected effect of epigenetic regulation? - Duration of the intended epigenetic regulation? - What is the target cell type for epigenetic effect? 2.2) Is a genetic modification necessary to establish the epigenetic effect?
Potential unintended effects of the used NPBT	1) Are genomic changes introduced at the modification site? 2) Are off-target modifications induced? 3) Are (epigenetic) effects on gene regulation induced? 4) Are non-plant sequences introduced into the breeding product? 5) Which kinds of uncertainties may be associated with the breeding techniques used? - Unintended phenotypical effects associated with NPBT? - Movement of novel molecules between plant parts - Adventitious reproductive functions established
Characteristics of the targeted traits	1) Source of trait 2) Function of trait(s) 3) Mode of action of trait 4) Type of trait 5) Stability of the trait

These considerations were derived from previous work concerning NPBT-crops (e.g. as referenced above) and from the existing guidelines for the risk assessment of GM-crops (e.g. EFSA 2010). Thus table 2 is presenting an overview on the approach used in the following subchapters to identify characteristics of individual NPBTs, which are considered relevant for the problem formulation for the respective risk assessment. Problem formulation is considered a crucial initial step in risk assessment and required to derive an appropriate “framing” of the risk assessment, identifying which qualitative risk issues are assessed in its course (cf. e.g. EFSA 2010, WOLT et al. 2010, HILBECK et al. 2011).

The above scheme was adapted from the current approaches to the risk assessment conducted for GM-crops (e.g. EFSA 2010). It is further integrating preliminary information available as regards the potential effects of NPBT-crops in comparison to GM-crops and crops developed by conventional breeding techniques. It will be used as a general outline for the discussion of specific characteristics of NPBT-approaches presented in the following chapter (Chapter 2).

It needs to be underlined that not all of the above considerations are relevant for all types of NPBTs and the resulting NPBT-crops.

For example certain types of approaches, like MAS, are not intended to introduce genetic or epigenetic changes other than those which can be expected for conventional breeding using available plant varieties of a crop species. Therefore only relevant considerations for a specific NPBT, i.e. a subset of the above list, may apply for a specific risk assessment exercise.

Breeding approaches may also be based on a combination of techniques, rather than on one NPBT alone, including combinations of different NPBT-approaches as well as combinations of NPBTs with GM-technology as indicated above. In most breeding approaches also conventional steps of breeding will be involved.

Some NPBTs furthermore require application of additional methods, e.g. in vitro propagation of cells, regeneration of plantlets from modified cells. These techniques have their own potential to introduce unintended effects, which needs to be taken into account when assessing the risks associated with a specific NPBT-crop. However such considerations – and the respective potential for adverse effects - will not be specific for NPBTs, as such methods are also commonly used in certain conventional breeding programmes and GM approaches.

Additionally it should to be noted that some considerations necessary for risk assessment are not at all related with the NPBT-technology involved, but result from the type of trait that is targeted by the breeding approach. The resulting trait characteristics may be comparable with crops developed by alternative breeding approaches, e.g. GM-crops or crops developed by conventional breeding. As an available example new crops which are tolerant to certain herbicides were developed by all of the above mentioned breeding approaches (NPBT, GM, conventional breeding). Adverse effects of such crops which are associated with the HT-trait and the changes of agronomical management necessary to exploit the HT-trait in cultivation would be comparable and not specific for the respective HT-NPBT-crop.

However any comprehensive approach to risk assessment thus needs to be designed to assess the full scope of risk issues (potential adverse effects which may be associated with a specific crop). Therefore assessment of effects related to specific characteristics of NPBTs need to be complemented by additional considerations, addressing the issues outlined above as well as consid-

erations addressing characteristics of the crop plant species, which is used for breeding, and relevant characteristics of the receiving environment of a specific application. Such considerations are commonly implicated in the current frameworks for the risk assessment conducted for GM crops and are also highly relevant for the risk assessment conducted for NPBT-crops. These considerations will not be the focus of the discussion of individual NPBTs in the following chapters. This study however highlights such issues if existing examples of NPBT-crops or those under development would indicate the specific need to address such aspects and any resulting adverse effects. A table which is integrating all considerations and provides additional detail to the specific issues is annexed to this report (Annex 2).

2 CHARACTERISTICS OF SELECTED NEW PLANT BREEDING TECHNIQUES

In plant breeding an increased phenotypic variability is tried to be achieved through recombination of genomes, traditionally in particular via crossing of different phenotypes (i.e. hybridization), or more recently through more directed changes at molecular level resulting from advances in molecular biology. After this step of creating genetic variability, it is necessary to select those phenotypes which display the desired traits.

In recent years the development of in vitro techniques (e.g. tissue culture techniques, protoplast fusion, embryo rescue) and the application of tools of molecular biology in plant breeding (e.g. marker assisted selection (MAS), quality traits loci (QTL) mapping and genetic transformation) opened new possibilities for more targeted breeding. Even deliberate changes in the regulation of existing genes are possible making use of the mechanism of RNA interference. In the following chapters selected new plant breeding techniques are briefly described taking into account the considerations summarised in Tab. 2. As far as possible each characterisation of the respective new plant breeding techniques is followed by a short description of the intended and potential unintended modifications and of the characteristics of traits targeted by examples of application of the NPBT. In addition issues relevant for risk assessment are presented, with a focus on aspects that are related to the changes associated with by a respective technique. The differences in risk relevant issues are due to the different aspects of a breeding process which are targeted by a specific NPBT and the different levels of organismic complexity which are targeted (see Tab. 3 for overview).

Creation of Genetic Variability	Creation of epigenetic Variability	Selection	Multiplication
Transgenesis	RNA-directed DNA-Methylation (RdDM)	Marker assisted selection (MAS)	Cell and tissue culture techniques
Cisgenesis/ Intragenesis		Proteomics/Metabolomics	Generative Multiplication
Nuclease-mediated site-directed mutagenesis		<i>In vitro</i> selection	Vegetative Multiplication
Oligonucleotide-directed mutagenesis		Agroinfiltration	Grafting
Cell fusion techniques		Phenotypic selection under controlled conditions (e.g. infection with pathogens)	
Mutagenesis/Tilling		Phenotypic selection in the field	

Table 3:
Individual NPBTs target different aspects of breeding processes:

2.1 Cell Fusion

First cell fusion, also called somatic hybridization, was believed to revolutionize plant improvement research. These expectations could not be met due to the difficulties associated with this technique (e.g. protoplast isolation, poor regeneration, elevated ploidy level of somatic hybrids). However in specific cases the application of cell fusion is successfully applied in variety improvement as it allows for overcoming crossing barriers and for new cytoplasm-nucleus combinations.

The Concept of Cell Fusion

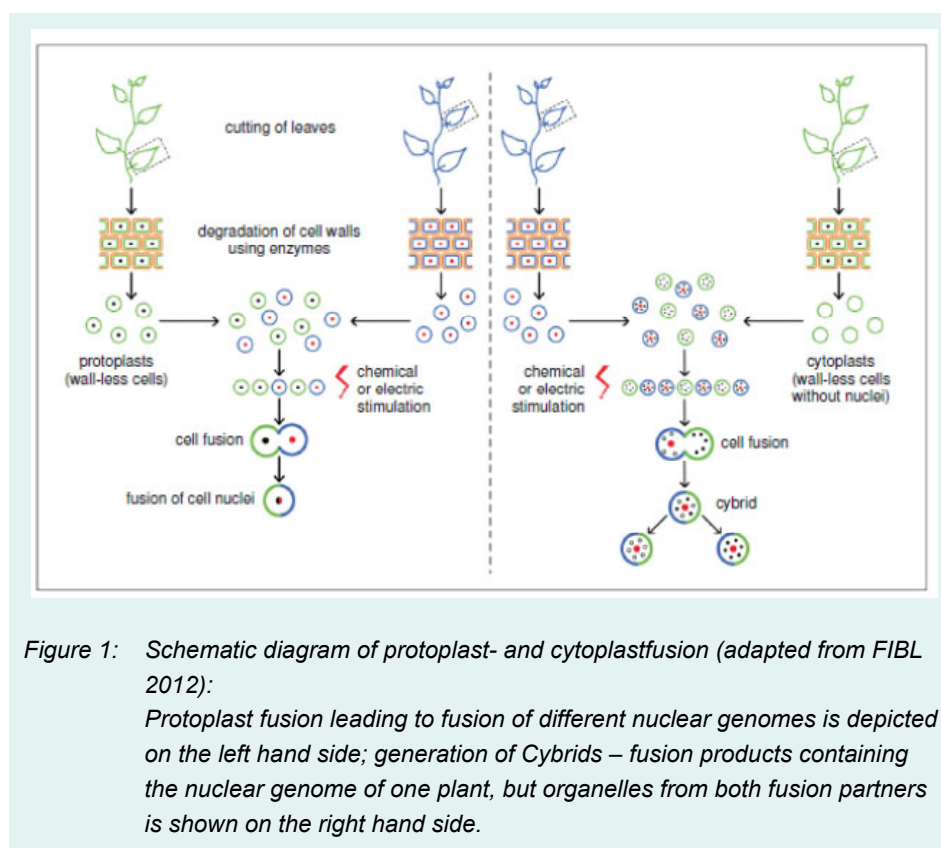
The fusion of two somatic cells is generally termed cell fusion. For plants the term somatic hybridization is also used as general term. In principle two wall-less cells (i.e. protoplasts) are fused in vitro to produce a hybrid cell. For the degradation of the cell wall various enzymes (e.g. cellulose, pectinase) are used. Usually fusion is induced by chemical (e.g. with polyethylene glycol) or electric stimulation. Then using hormones the formation of a cell wall is induced in the somatic hybrid cell. The hybrid cells are then grown into calluses from which plantlets are regenerated and finally grown to a full plant (i.e. somatic hybrid). However the regeneration of plants from protoplasts is generally problematic and the selection of hybrid cells from un-fused or self-fused parental protoplasts is also associated with difficulties (BROWN & CALIGARI 2008).

In contrast to the natural occurring fusion of two gametes (i.e. sexual hybridisation) where only the maternal cell organelles (e.g. mitochondria, chloroplasts) are inherited, in somatic hybrid cells the cell organelles of both parents are recombined when multiplied and regenerated.

Depending on whether both parental cells contain nuclei or not two types of cell fusion can be distinguished in plants:

- Protoplast fusion (i.e. somatic hybridisation)
Nuclear and cytoplasmatic genes are recombined by the fusion of two somatic cells. When cell fusion is followed by fusion of the two nuclei the somatic hybrids will have the combined chromosome number of both parents (i.e. (allo) polyploidy). Depending on the species either the chromosome number of the parents or of the hybrid combination might have to be reduced (e.g. using colchicine).
- Cytoplasm fusion (i.e. asymmetric cell fusion)
If the nucleus of one of the cells is destroyed prior to fusion with another cell, only the extra-chromosomal DNA of the cell organelles is transferred without changing the nuclear DNA

According to Annex I A, Part 1.3 of EU Directive 2001/18/EC cell fusion (including protoplast fusion) is considered a technique of genetic modification (EC, 2001). Only if the organisms involved can also exchange genetic material through traditional breeding methods according to Annex I.B cell fusion (including protoplast fusion) is exempted from the Directive (EC, 2001).



2.1.1 Intended Modification

In somatic hybridization (i.e. protoplast fusion) the nuclear DNA as well as the extrachromosomal DNA of the cell organelles is recombined. During regeneration and replication of the somatic hybrids chromosomes and cell organelles of both parents will be rearranged and multiple new combinations may be formed. In addition the fusion of two somatic cells enables the recombination of genomes from plants which cannot reproduce sexually. Interspecific (*Solanum tuberosum* and *Solanum bulbocastanum*) or even intergeneric (between *Lycopersicon* and *Solanum*) hybrids may be produced (WOLTERS et al. 1994). In the latter case however there is an increased chance for hybrids to be infertile.

Advancement in cell fusion techniques has been achieved with so called cytoplasm fusion (CF), also called asymmetric cell fusion. With this approach a more direct combination of maternally inherited traits like cytoplasmic male sterility (CSM) with chromosomal traits is achievable. Cytoplasts are protoplasts in which the cell nucleus has been destroyed (e.g. via X-ray). A cytoplast can be fused with a protoplast to form a cytoplasmic hybrid (i.e. cybrid). CF is applied to specifically combine traits located in plastids or mitochondria (e.g. CMS) or to integrate single resistance genes from wild relatives into cultivated varieties e.g. of potato or rice (Messmer et al. 2012). As no nuclear chromosomes are transferred, no other but the desired traits located in the cell organelles are being introduced. This excludes potential unwanted recombination events between chromosomes and thus reduces the necessity for backcrossing.

2.1.2 Potential Unintended Effects of the Modifications

In cell fusion techniques the recombination of the chromosome is incidental and not only the desired trait will be integrated, but also many other unwanted genetic elements. This however implies a lot of backcrossing efforts before an elite variety is regained.

Cell fusion techniques are rather complex and are associated with various drawbacks, ranging from a low frequency of regenerated and fertile hybrids, to somaclonal variation caused by tissue culture. In addition gene regulation between the nuclear genome and the extrachromosomal DNA may be affected which may lead to unintended effects. Most importantly unpredictable chromosomal rearrangements resulting from the fusions of two cell nuclei are manifold, chromosomal eliminations occur frequently and genetic instability is widespread. Thus a careful selection procedure and backcrossing steps following the modification are indispensable when applying cell fusion.

2.1.3 Characteristics of the new traits from examples of plants obtained with cell fusion techniques

Currently protoplast fusion is mainly used in the development of biotechnological applications involving fungi (e.g. *Streptomyces* sp., *Trichoderma* sp.) and bacteria (VERMA et al. 2008). In plant breeding intraspecific cell fusion is not widespread, but for instance applied in vegetable breeding. Somatic hybrids have for instance been used to introduce disease resistance genes from sexually incompatible wild species to rice and potato varieties (Helgeson et al. 1998). Additionally somatic hybridization is used in citrus scion and citrus rootstock improvement (GROSSER et al. 2000).

Cytoplasm fusion is in particular applied, if traits which are bound to cell organelles, that are inherited maternally, are to be combined with chromosomal traits (MESSMER et al. 2012). This is for instance the case with CMS, which is a genetic defect located at the mitochondrial DNA producing male sterile plants. This trait is of high importance for the production of hybrid seed as there self-fertilization of the parental inbred lines must be avoided. Therefore protoplast fusion is for instance widely used in vegetable breeding to introduce CMS from radish into cabbage species (e.g. white cabbage, cauliflower, broccoli, cabbage turnip) (THOMMEN 2008).

2.1.4 Risk relevant issues

In cell fusion techniques the resulting changes in the genome are more profound than in conventional cross-breeding as for instance they involve changes in the number of the chromosome set (i.e. ploidy level) as well as recombination of extrachromosomal DNA. Additionally the genomic changes depend very much on which parts of the genome are fused. If two protoplasts are fused including the fusion of their nuclei, chromosomal rearrangements may be substantial and the resulting genome may be altered substantially compared to the “parental” genomes. If cytoplasm fusion is applied not the genome of the cell nuclei but only the genetic information of the cell organelles (e.g. plastids) is altered. In any case random changes take place which are more frequent and more intense

than with natural recombination in cross-breeding. In breeding in general plants with elevated number of chromosomes are often associated with increased vigour which may have consequences for persistence and invasiveness of the plants constructed with this technique if the increased chromosome number is not deliberately being reduced.

2.2 Marker Assisted Selection (MAS)

In order to select those phenotypes which display the desired trait a selection procedure is indispensable following creation of variability (via hybridization or mutagenesis) in – or following genetic transformation of – a target organism. Traditionally in plant breeding morphological markers are being used (e.g. pigmentation, dwarfism, leaf shape) in the selection of desired individuals. With the introduction of in vitro techniques (e.g. cell and tissue cultures) marker systems which allow for selection early in plant development before the final phenotype has been developed are needed. Advances in biotechnology enabled the development of more efficient selection systems (e.g. biochemical or molecular marker systems) replacing traditional phenotype-based selection systems.

Any selection system that relies on the indirect selection of traits of interest through markers linked to them can be referred to as marker assisted selection (MAS). The most widely known example for marker systems is the use of selectable markers in genetic transformation. There usually antibiotic or herbicide resistance genes are introduced into the recipient organisms together with the, usually qualitative, trait of interest. Successfully transformed genotypes survive the application of herbicides or antibiotics while those which do not contain the recombinant DNA are eliminated. In addition markers appropriate for screening are being developed, which allow the identification of those genotypes which contain the desired trait without destroying the others. Most MAS applications use genetic screening markers rather than phenotypically selectable markers.

Nowadays MAS refers in particular to selection based on genetic information retrieved through the application of molecular markers (ASINS et al. 2010). Molecular markers make differences in the DNA sequence visible, which can be related to different phenotypes. So in breeding programmes molecular markers are used to select for traits at the DNA level. On the one hand they facilitate the choice for the elite parental lines to be used in cross breeding and on the other hand the decision on which offspring to continue breeding with or to choose for multiplication (i.e. seed production).

So MAS can be very useful to efficiently select for traits that are difficult or expensive to measure or are expressed late in development. This is particularly relevant for crops with long-lasting juvenility (e.g. trees species) as selection is facilitated already at the seedling stage. So MAS is most frequently used to eliminate disease susceptible genotypes or to introgress disease resistance genes into well-adapted elite lines early in the breeding programs (ASINS et al. 2010). MAS can be particularly useful in pyramiding monogenic resistance gene, which cannot be distinguished by phenotype (MESSMER et al. 2012).

However breeding objectives in cross breeding not only involve monogenic (qualitative) traits, but often involve complex traits (e.g. disease or pest resistance). These so called quantitative traits are influenced/specified by various genes (polygenic effect). The respective phenotypes vary in degree and unlike discrete characteristics are measurable on a continuous scale. Thus the challenge is the identification of markers linked to the respective quantitative trait. In other words marker loci need to be identified which lie in close proximity to those loci on the chromosome determining the quantitative trait. As molecular markers are inherited according to Mendelian laws quantitative trait loci (QTL) analysis can be used for this purpose. QTL analysis comprises the joint study of the segregation of marker genotypes and of phenotypic values of individuals or lines which enables the location and effect-estimation of the genetic elements controlling a trait of interest (ASINS et al. 2010). Once the relationship between molecular markers and the desired trait is established, MAS can remarkably assist breeding programmes.

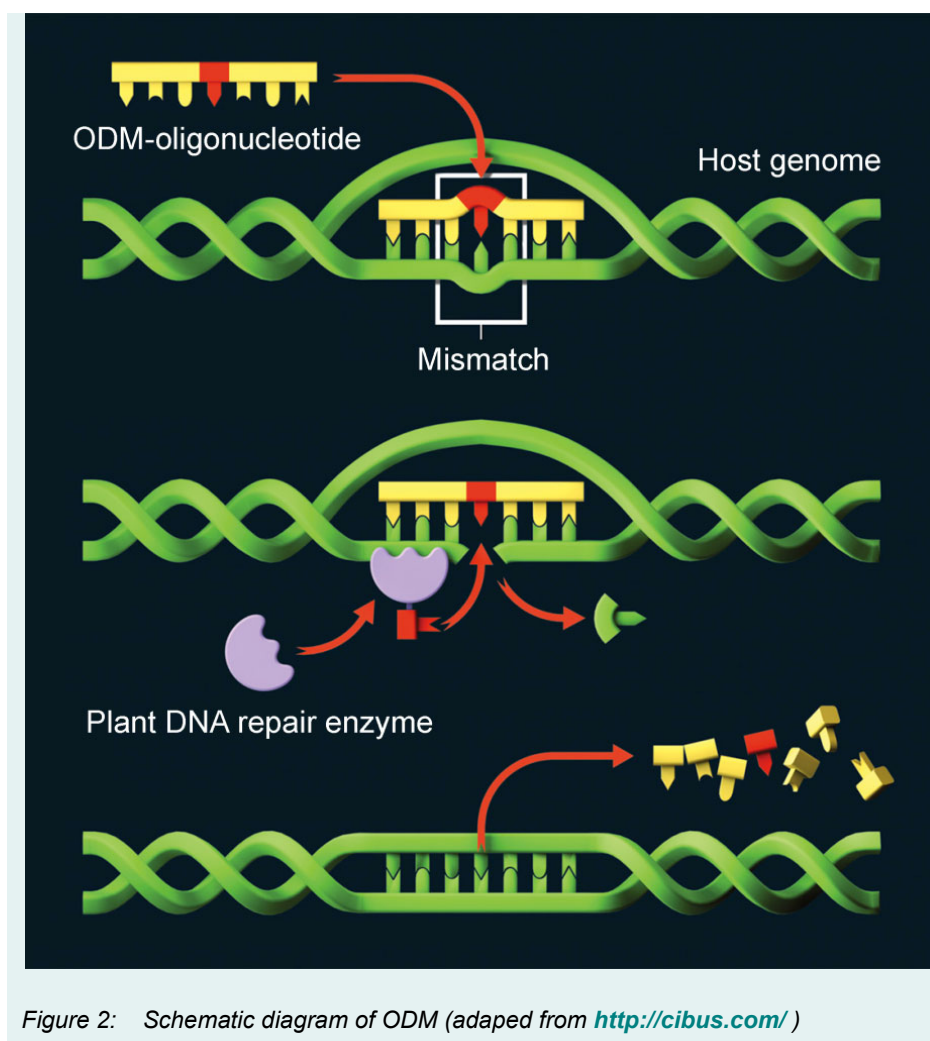
MAS aims at deepening the understanding of the specification of qualitative traits at a molecular level. Applying MAS does in no way alter a plant's genetic configuration. Thus the paragraphs above do not follow the structure chosen for the other new plant breeding techniques discussed in this report. However it is worth noticing that with MAS the focus of selection criteria applied in plant breeding is shifted towards the DNA level and disregards genotype-environment interactions as well as epigenetic effects.

2.3 Oligo-directed mutagenesis (ODM)

In addition to mutation-breeding by random mutagenesis the development of methods to introduce mutations in a targeted way was started in the 1970s (cf. LUSSEY et al. 2013). Oligo-directed mutagenesis (ODM) – also known by a number of different names (see e.g. LUSSEY et al 2011 and BREYER et al. 2009) - was one of the first approaches to implement such a concept in plant breeding in the late 1990s.

The Concept of ODM

ODM is exploiting the finding that oligonucleotides of short or medium sized sequence length, can be used to induce mutations at genomic DNA sequences, which are complementary to the oligonucleotide sequence except for single or very few positions. Upon introduction of the respective oligonucleotides into target cells they associate with complementary genomic sequences, thereby creating sites of sequence mismatch(es). During subsequent steps of DNA replication mutations can be introduced at the mismatched positions. By this process intentional sequence changes at specific nucleotide positions can be introduced into the genomic target sequences, which are directed by the nucleotide sequence of the synthetic oligonucleotides used in ODM (cf. BREYER et al. 2009). This technique is applicable to introduce targeted mutations into the genomes of microorganisms, animal and plant species using a similar general approach as depicted below.



2.3.1 Technical Details of ODM

ODM employs different types of oligonucleotides for a targeted induction of point mutations at specific sites in the DNA sequence of a plant genome (for review see e.g. NTWG 2011; LUSSEER et al. 2013, WALTZ 2012). Such oligonucleotides can either be in vitro synthesized single stranded DNA oligonucleotides or chimeric oligonucleotides including DNA and RNA bases, RNA-oligonucleotides or oligonucleotides consisting of nucleic acid analogues (NTWG 2011). These oligonucleotides are designed to share sequence homology with certain target sequences of the plant genome with the exception of one or a few base pairs thus intentionally creating sites of sequence mismatch. Such oligonucleotides with a size of approximately 20 to 100 nucleotides are then delivered to target cells by methods suitable for the different cell types including e.g. electroporation, transfection mediated by polyethylene-glycol or natural cellular uptake mechanisms. Due to their sequence homology they associate with the genomic target sequences and induce site-specific mutations via the natural DNA repair mechanisms operating in the targeted cells. These repair mechanisms are triggered by the sequence mismatches between the oligonucleotide- and genomic sequences. Commonly mismatches of a length of 1-4 nucleotides are used in ODM (see LUSSEER et al. 2011). During DNA repair site-specific nucleotide sub-

stitutions directed by the used oligonucleotide sequence are introduced in a fraction of the dividing cells due to activation of cellular systems for mismatch repair or nucleotide excision-repair (BREYER et al. 2009). Such changes can lead to intended induction of a point mutation or the reversion of an existing mutation present in the target crop genome. LUSSEY et al. (2012) also take note that occasionally other sequence modifications, e.g. deletions or insertions at the genomic site of modification, can result from ODM.

2.3.2 Intended modifications

As described above ODM may be used to create specific changes in a known part of the genomic DNA sequence of a target crop. In comparison to random mutagenesis by means of chemical mutagens or physical inducers of mutagenesis, e.g. radiation, properly designed ODM approaches achieve a high specificity of the mutations, i.e. highly efficient targeting (Breyer et al. 2009). However a quite stringent limit is noted regarding the number of targeted nucleotide changes in a single ODM experiment. Only a single or a few nucleotide changes (max. up to 4) are possible by one-time ODM (for review see e.g. AGES 2012).

The success of an ODM experiment (intended targeting, sufficient efficacy) is dependent on a number of factors, like:

- Design of the oligonucleotide sequence used for ODM (Length and sequence of oligonucleotide selected for ODM, number and location of target sequence mismatches).
- Type of the oligonucleotide used (DNA, RNA, chimeric oligonucleotides, oligonucleotides with chemical modifications).
- Efficiency of transfection procedure and of oligonucleotide uptake into target cell nucleus.
- Type of target cell (species, tissue) and developmental status of the targeted cell.
- Efficacy of regeneration of target cell line and of the selection regime employed, if any.

Several limitations of the ODM technique are associated with the above listed factors. First of all the target sequence must be known to support the design of an appropriate oligonucleotide. The length of such oligonucleotides must strike a balance between increased stability of the resulting complexes with the target sequence for longer oligonucleotide and detrimental effects associated with longer oligonucleotides, i.e. increased toxicity to target cells and a lower level of delivery to the nucleus (cf. AGES 2012). Chimeric or chemically modified oligonucleotides are characterized by a higher stability in the target cells, thus increasing efficacy of mutagenesis.

However it was noted, that neither the efficiency nor the specificity of the ODM technology can be sufficiently controlled. The efficiency for inducing specific mutations in plant cells is lower than for other target cells, e.g. animal cells (see discussion in AGES 2012). Availability of an efficient selection regime is thus helpful to retrieve plants with intended phenotypes. This however limits the range of possible applications (see also chapter 2.3.4).

ODM is intended to modify either the DNA sequence of a specific gene which may lead to changes in the function of the gene product or to modify the expression of a specific plant gene present in the genome of the target crop (NTWG 2011). Silencing of genes can be achieved by creating gene knock-outs, e.g. through introduction of stop codons, frameshift mutations or deletions interrupting the reading frame of the target gene (KMIEC et al. 2003).

2.3.3 Unintended modifications

ODM is generally considered to introduce mutations in a more targeted way than other mutational techniques (random mutation) (BREYER et al. 2009). However certain possibilities to introduce unintended effects are associated with the method:

- Semi-targeted, non-specific mutations were also observed for ODM (see e.g. BRITT & MAY 2003; KOCHVENKO & WILLMITZER 2003). Such mutations were observed in nucleotides adjacent to the intended target site (SCHAART & VISSER 2009).
- Knock-out mutations, which result in expression of fusion genes, should be assessed for potential adverse effects of their products.
- Sufficient partial homologies of off-target genomic sequences with the ODM-oligonucleotide can lead to mutations created at other sites in the plant genome than the targeted site. This is particularly relevant for target sequences which are repetitive in the genome (e.g. repeated motifs in regulatory sequences or structurally related genes). Sufficient knowledge of the (full) genomic sequence of a target crop species is therefore required to comprehensively assess such a possibility. Off-target effects may also not be easy to anticipate, as single mutations can have relevant effects, e.g. lead to an increase in expressed plant toxins (KUZMA & KOKOTOVICH 2011).
- In comparison with GM technology no vector sequences or other foreign DNA sequences are introduced (BREYER et al. 2009), however similar transfection methods are used for introduction of the ODM-oligonucleotides into the target cells. These methods (e.g. transfection mediated by chemicals, biolistic bombardment) are themselves associated with a potential to elicit unintended mutations (WILSON et al. 2006).
- Extended stability of oligonucleotides used in the transfected cells during ODM can also lead to unintended effects. Chimeric or chemically modified oligonucleotides however are specifically used because of their increased stability leading to better performance. An improved knowledge on the degradation kinetics of ODM-oligonucleotides would be necessary to assess effects due to oligonucleotide stability.
- In addition to introducing point mutations the oligonucleotides used in ODM can also integrate into the genomic plant DNA, similar to integration of transgenic DNA. The frequency of integration is higher for oligonucleotides with increased number of consecutive mismatches as compared to target sequences (SAWITZKE 2013).
- Eventually, ODM oligonucleotides may trigger the regulatory RNAi-machinery leading to unexpected regulatory changes in cellular gene expression (HEINEMANN et al. 2013).

2.3.4 Characteristics of the new traits from plants obtained by ODM

Unlike other targeted mutation technique ODM was shown to be applicable for a wide range of relevant crop species (LUSSEER et al. 2011). Plant species subjected successfully for ODM include e.g. maize, wheat, canola and banana. A focus of these developments was the introduction of traits for establishing tolerance of the engineered crops to certain broadband herbicides. Herbicide tolerance (HT) as a target trait is associated with the advantage that the trait itself can be used for efficient selection of cells and plants carrying the intended mutation.

However ODM is also applied to introduce other traits than HT, such as prolonged shelf life, pest resistance and for improving quality and health features and yield (for a review see LUSSEER et al. 2011).

Crops with these non-selectable traits may be obtained by using high throughput methods for screening of potential mutants, e.g. high throughput sequencing (LUSSEER et al. 2011). It is expected that increased implementation of such methods will aid the developments of crops with non-selectable traits by ODM.

2.3.5 Risk Relevant Issues

As regards molecular changes potential effects may either be due to the effects of the mutation(s) introduced by ODM or result from the technique used to introduce the ODM oligonucleotides into the target cells. Furthermore the techniques necessary to regenerate plantlets from mutated cells have a potential for unintended effects.

Through ODM known sequence elements (genes) should be mutated in a targeted way. These mutations can either be point mutations of genes which result in desired changes of gene product function(s), or knock-out mutation which silence the expression of the targeted genes (loss of function). These changes are stably inherited in a similar pattern as comparable genomic elements; i.e. in a Mendelian way if nuclear genes are targeted by ODM or similar to mitochondrial or plastid genetic elements, if such elements are targeted).

Due to the targeting of mutations to specific genomic sites, which can be very efficient dependent on the ODM design and experimental protocol, less off-target mutations can be expected than seen with approaches to mutation breeding resulting in random mutagenesis.

However as discussed above some kinds of unintended modifications through ODM are possible, e.g.

- Off-target mutations in genomic elements sharing homologous sequences and unintended integration of whole or partial ODM oligonucleotide sequences.
- Expression of fusion-proteins for some types of knock-out mutations.
- Unintended modification due to transfection and regeneration methods.
- Unintended effects of ODM oligonucleotides in cellular regulation pathways for gene expression (e.g. RNAi).

Partly such unintended effects would be similar as for crops developed by GM technology, due to the fact that comparable methodological steps are involved (cf. chapter 2.3.3). Respective risk issues thus need to be addressed by a comprehensive molecular characterisation, taking into account the experiences with risk assessment of GMOs (EFSA 2010, 2011).

Regarding the risks associated by the engineered trait, it is evident that also for ODM case specific considerations will apply. These considerations depend on the available knowledge and previous experience with respective traits in crop development. While some ODM traits and the respective gene products will have a corresponding history of use in crop breeding and consumption this is certainly not the case for all engineered traits.

Some possible mutations will not exist in the current breeder's gene pool according to the definition of PODEVIN et al. (2012) for a specific crop. E.g. mutations may be modelled on the sequence of genes from a non-crop origin. Other mutations will be based on basic research findings rather than on experience of such traits in crop breeding. Introducing such mutations via ODM will be comparable to the transfer of "foreign" genes by GM technology – although the specific differences (e.g. potential different genomic location and/or regulatory context) need to be taken into account.

Accordingly the knowledge on the effects of specific mutations and on the function and effects of mutated gene products in the context of the genetic background of a specific crop will not be good enough to conclude on the safety of the respective NPBT-crop without further assessment. In this respect the overall framework for assessment of health and environmental risks as developed for GM crops (cf. EFSA 2010, EFSA 2011) can be used for guidance to develop an appropriate approach for crops with "new" traits developed by ODM.

Compared with the transfer of whole foreign genes, the changes introduced into endogenous genes by ODM are regarded to be quite small (e.g. with point mutations due to single nucleotide exchanges). However several issues need to be taken in consideration in this respect:

Even small molecular changes may result in pronounced effects on the expression of respective genes and/or their functions in a specific crop.

More extensive modifications may be due to certain ODM approaches (e.g. with several mutational cycles, developments utilizing integrated ODN oligonucleotides etc.)

Some agronomically important traits, e.g. HT-traits developed by ODM, are based on point mutations of genes involved in the metabolic pathways of herbicide action in crop cells. Nevertheless relevant risk considerations need to be addressed with such traits, as the (environmental) impacts of changes in agronomical management, due to exploitation of these traits during crop cultivation can be pronounced. Practical experience with similar traits developed by GM technology supports the argument that extensive (indirect) impacts can be associated with small molecular changes.

2.4 Nuclease-mediated site-directed mutagenesis

Another way to introduce mutations into the genomic sequence of crop plant genomes in a targeted fashion is based on the use of site-specific nucleases. Specific experimental approaches involving site-specific nucleases can also generate other genomic modifications, including introduction of foreign DNA sequences into the crop genome at specific genomic locations.

The Concept of Nuclease-mediated site-directed mutagenesis

Several types of synthetic site-specific nucleases (SSN) were developed in recent years for genetic modification of crop plants as well as other organisms (for review see e.g. PUCHTA & FAUSER 2013, VOYTAS 2013, GAJ et al. 2013). These techniques utilize different types of synthetic nucleases with the general aim to introduce double strand breaks at specific sites of the genomic DNA of the respective (crop) species. Such double strand breaks then trigger different DNA repair mechanisms, which are naturally operating in the (plant) cells: One of these repair mechanisms facilitates the repair of double strand breaks by non-homologous end joining (NHEJ). NHEJ may introduce random mutations (point mutations, Indels – deletions and/or insertions) at the target site (for additional details see 2.4.5. SSN1). In case an additional donor DNA is supplied, which is sharing homologies to the genomic sequences bordering the double strand breaks introduced by a SSN, other modifications may be introduced by homologous recombination (HR): In addition to introducing specific point mutations or the reversal of mutations present in the crop genome, also additional recombinant sequences can be introduced at a specific genomic location into the DNA of somatic or reproductive cells (PUCHTA & FAUSER 2013).

During recent years a number of different nuclease systems for facilitating such approaches have been developed. All of these nucleases are combining two functions: On the one hand they contain elements that recognize specific DNA sequences occurring in the genome of the targeted crop. Upon binding to such genomic target sequences these elements ensure a specific localization of the nuclease in the target genome. On the other hand they contain an enzyme domain, which is cutting both DNA strands at a precise location respective to the recognition sequence for localization (e.g. a site-specific nuclease). However due to their different origin and characteristics of the respective enzyme domains the following types of site specific-nucleases can be distinguished:

- Zinc-finger nucleases (ZFN)
- Transcription activator-like nucleases (TALEN)
- Meganucleases / Homing endonucleases (HEs)
- CRISPR/Cas-Nucleases (CRISPR)

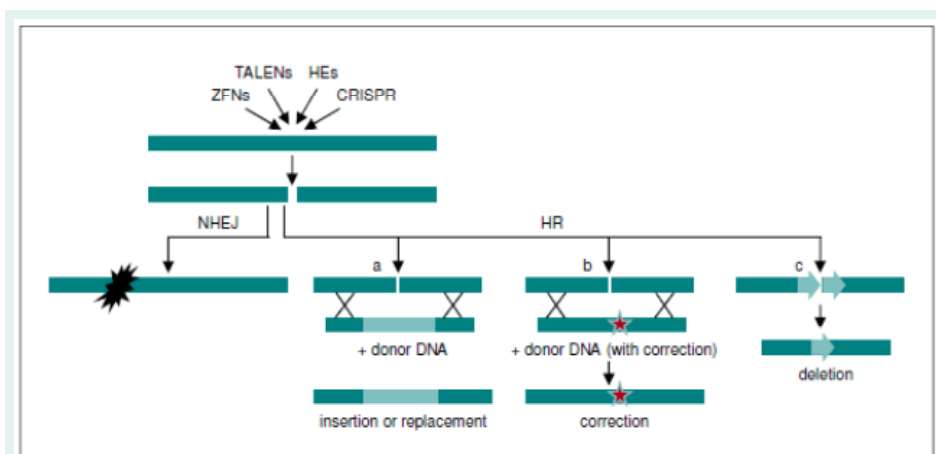


Figure 3: Schematic diagram of Nuclease-mediated site-directed mutagenesis (adapted from Pauwels et al. 2014)

NHEJ: DNA repair by non-homologous end joining, HR: homologous recombination;

HR mechanisms type a and b depend on presence of additionally introduced extrachromosomal DNA elements (donor DNA) directing the effect of HR, deletions in genomic DNA can be introduced by the mechanism depicted in c as well as by NHEJ events if two neighboring double-strand breaks are introduced and by mechanism b if the donor DNA is harbouring a deletion.

2.4.1 Zinc-finger nucleases (ZFN)

Zinc-finger nucleases (ZFNs) are protein dimers, which consist of two independent subunits. Each subunit is composed of a DNA-binding domain and a nuclease domain. Typically, a heterodimeric version of the endonuclease FokI is used as a nuclease in ZFNs. The DNA-binding domains are composed of Zinc-Finger (ZF) DNA-binding domains linked together into arrays of 3-4 ZFs. ZFs are finger-like structures, which recognise specific stretches of three nucleotides (nt) in a DNA sequence. Since each zinc finger recognizes 3 nt, a ZF-domain is targeting a 9–12 nt long DNA recognition site.

2.4.2 Transcription activator-like nucleases (TALEN)

TALENs are dimeric enzymes with a structure which is related to ZFNs, i.e. composed of a nuclease domain fused to a DNA-binding domain. Similar to ZFNs, FokI is usually used as a nuclease domain. However the DNA-binding domain of TALENs is more flexible because it consists of modules recognizing single nucleotides in a DNA sequence. The DNA-binding domain then consists of an array of up to 30 modules, which are specific for a particular nucleotide sequence of 30 nucleotides. Due to their longer DNA recognition sites TALENs are more specific for particular genomic locations and thus cause fewer unwanted off-target effects than ZFNs. TALEN approaches were also applied for modification of plant genomes (Li et al. 2012).

2.4.3 Meganucleases / Homing endonucleases (HEs)

Meganucleases are naturally occurring, rare cutting endodeoxyribonucleases that are characterised by a DNA recognition site of typically 20–30 nucleotides (see. PUCHTA & FAUSER 2013, VOYTAS 2013). They were isolated from mobile introns of a wide range of organisms, including the yeast *Saccharomyces cerevisiae*, green algae (e.g. *Chlamydomonas reinhardtii*) and archaeobacteria (e.g. *Desulfurococcus mobilis*). These small enzymes, which can be composed of a single monomer or a dimer, consist of a central nuclease domain flanked by two DNA binding domains, which are recognizing a specific DNA sequence. This enzyme thus functions as a target specific nuclease with a pattern of genomic cleaving sites dependent on length and nucleotide sequence of the target sequence. Due to the limited number of different MN/HEs, a limited repertoire of naturally occurring recognition sequences is available. However, the sequence specificity of naturally occurring MN/HEs may be modified by mutation of the natural recognition domain, or by fusing recognition domains from different enzymes. With such approaches a variety of different MN/HEs was developed in recent years (e.g. PODEVIN et al. 2013, ANTUNES et al. 2012, GAO et al. 2010, TZFIRA et al. 2012).

2.4.4 CRISPR/Cas-Nucleases

CRISPR/Cas nucleases are synthetic nuclease complexes, developed from the bacterial nuclease Cas9 (CRISPR associated 9), which is a component of the adaptive immunity system in bacteria aimed to recognize and destruct foreign DNA, e.g. phage DNA or plasmid DNA. Recently CRISPR/Cas-based nucleases are also applied in targeted editing of crop plant genomes (e.g. Shan et al. 2013, Mao et al. 2013). CRISPR/Cas nucleases are guided to a particular genomic DNA sequence by guide RNAs attached to the nuclease enzyme. A naturally occurring model for such guide RNAs is provided by the RNAs directing Cas9, e.g. a complex between CRISPR-RNA (crRNA) and transactivating crRNA (tracrRNA) (PUCHTA & FAUSER 2013). However the enzyme also accepts specifically designed synthetic guide RNAs modeled on the Cas9 guide RNA. These synthetic guide RNAs direct the nuclease activity to intended target sequences in the crop genome, which are complementary to the synthetic recognition sequence of the guide RNA. By this way a multitude of different target sequences and thus different genome sites can be targeted.

2.4.5 Intended modifications by site-specific nucleases (SSN)

The types of modifications introduced in crop genomes can be quite different, dependent on the type of SSN used for modification and its specific target sequence(s) in the crop genome. Furthermore the outcome of the modification depends on the design of application of a SSN: I.e. which type of repair mechanism or recombination is activated by the SSN and which additional DNA templates (if any) are supplied during a particular experiment.

In analogy to the classification used for ZFNs by the EU Working Group on New Techniques (NTWG 2011), the possible modifications by SSNs may be categorised in three classes, SSN1-3 (cf. PODEVIN et al. 2013):

SSN1:

The SSN1 mechanism can generate site-specific random mutations at the genomic site(s) harboring the nuclease recognition sequence. Single double strand breaks in the genomic DNA are repaired mainly by non-homologous end joining in somatic plants. This mechanism may result in “unfaithful” repair of the sequence present at the double strand breaks created by a SSN. Thus small nucleotide deletions and/or insertions result which lead to either point mutations in the targeted genes or to deletion of sequences from specific plant genes, which prevent that functional gene products are expressed. Thus SSN1 may create targeted gene knock-out mutations.

However SSN1 can also be designed to introduce neighboring double strand breaks, causing a deletion of the region between the two target sites (e.g. PACHER et al. 2007). Deletions of different length may be achieved, e.g. to delete regulatory regions, exons/introns, whole genes or even parts of chromosomes. Also duplications and inversions of the sequences located between the two double strand breaks, as well as the induction of translocation events between chromosomes (see review by PODEVIN et al. 2013).

SSN2:

SSN2 applications depend on availability of an additional DNA template (donor DNA) containing a sequence with high homology to the target site which is comprising the desired mutations. This mutated sequence is then functioning as template sequence for the repair process that substitutes the original endogenous sequence by the donor DNA sequence introducing the desired mutations. Thus targeted repair of mutations residing in the crop genome can be achieved, or additional mutations or even gene alleles can be introduced. Expression of the modified genes can then elicit the desired phenotype(s).

SSN3:

SSN3 similar to SSN2 is dependent on homologous recombination between sequences flanking double strand breaks and donor DNA. However with SSN3 approaches additional DNA elements, e.g. additional genes or transgenes are integrated. In comparison to conventional GM technology the transgenic DNA is thus integrated into a specific target location. This way precise localization of a transgenic construct in the genome of the modified crop can be assured, whereas in GM technology the transgenic DNA elements are usually integrated at random sites.

2.4.6 Unintended effects

Approaches to targeted mutagenesis by SSNs are subject to a number of possible unintended effects. According to the current lack of knowledge on the details of the involved mechanisms, significant uncertainties are associated with an assessment of unintended effects.

- Targeting of the SSNs might be not sufficiently specific: Use of not fully customized SSNs is associated with the possibility that the utilized SSN will introduce secondary off-target double strand breaks, which also trigger mutagenesis at these sites.

The different classes of SSNs are characterized by different natural specificities – MN/HEs are generally characterized by a high specificity and thus a

lower frequency of double strand breaks at off-target sites, whereas a lower specificity has been observed with ZFNs (as compared with MNs and TALENs). ZFNs are resulting in significant off-target activity and therefore higher levels of cellular damage (PODEVIN et al. 2013). Also CRISP-Cas systems can lead to off-target effects (Fu et al. 2013, Hsu et al. 2013). The issue of off-target effects needs also to be taken into account with SSNs (EFSA 2012b). This issue is particularly relevant when SSNs are used, which contain synthetically designed sequence recognition-domains.

- Even if targeting is specific, the outcome of repair at the double strand breaks induced by SSNs can be very diverse (e.g. point mutations, sequence/gene deletion, integration of non-native sequences, inversions/translocations of chromosomal sections). According to the nature of the outcome, an appropriate range of unintended effects need to be taken into account. The uncertainty concerning potential unintended effects will increase with the breadth of the genetic changes introduced.

- Knock-out mutations, which are the result of deletions leading to e.g. frameshift-mutations, might lead to expression of fusion genes, potentially associated with adverse effects.

- Additional sources of potential unintended effects are associated with a need to deliver functional SSNs and the donor DNAs for SSN2&3s to the target crop cell to conduct gene targeting via SSNs.

Usually genes encoding the SSN and the donor DNA are introduced transiently or stably into the target plant cells and the respective genes are then expressed to functional enzymes.

Gene delivery into the plant cell is achieved via various methods: For transient expression SSN genes and donor DNA are co-delivered by different methods: including electroporation, Agrobacterium mediated transformation and biolistic transfection of expression vectors for the SSN and the donor DNA. An alternative is stable transformation of plant cells with expression constructs using GM technology, leading to integration of the SSN-encoding gene into the host genome. After targeted mutagenesis (e.g. targeted integration of the transgene contained in the donor DNA, the integrated SSN-transgene is removed by segregation (see review by EFSA 2012b).

Thus at an intermediary step of the procedure methods of GM technology are involved, which can result in unintended modifications to the crop cells associated with such methods.

- The expressed nucleases themselves can exert adverse effects upon expression in the target cells (cf. SZCZEPEK et al. 2007).
- As noted by EFSA (2012b), the whole procedure includes phases when crop cells are propagated in tissue culture. As with other breeding techniques, cell propagation in tissue culture may induce unintended changes in the crop genome by somaclonal variation.
- Dependent on their nucleotide sequence, guide RNAs of CRISPR/Cas nucleases might trigger unintended effects on regulation of cellular gene expression by the RNAi-system (HEINEMANN et al. 2013).

As noted by EFSA (2012b), a high overall similarity of issues considered with transgenic, intragenic or cisgenic approaches exists (specifically with respective SSN3 approaches). EFSA also notes that some sources of unintended effects might be more relevant for either standard GM technology (i.e. effects due to

random integration at nonspecific target sites) or mutation breeding (i.e. effects due to untargeted random mutagenesis). However the assessment of unintended effects due to SSN approaches is considered a relevant issue.

2.4.7 Characteristics of the new traits from plants obtained by site-specific nucleases

Several SSN approaches were recently applied for modification of plants (see e.g. PUCHTA & FAUSER 2013), a couple of these applications were also targeting important crop species, among them maize, soybean and oilseed rape (cf. LUSSEER et al. 2011; PODEVIN et al. 2013).

Most of these applications were initially based on ZFN technology, however it can be expected that the other SN techniques will also be pursued in the future as alternative options for development (cf. LUSSEER et al. 2012). A recent review by PODEVIN et al. (2013) identified that by such approaches a number of different traits are targeted, involving modification of a range of target genes as e.g.

- herbicide tolerance (acetolactate synthase gene);
- virus resistance (translation initiation factors);
- lowering anti-nutritional compounds, e.g. erucic acid content in Brassicas (fatty acid elongases) and allergen content in peanuts (conglutin gene);
- improved nutritional value via elevated levels of carotenoids and modification of the carotenoid balance (zeaxanthin epoxidase) ;
- modified starches and fats for food and non-food uses (starch synthases, branching enzymes, and fatty acid desaturases) ;
- longer shelf life (e.g. aminocyclopropane (ACC) oxidase and polygalacturoanase);
- improved quality by reducing enzymic (polyphenol oxidases) and nonenzymic browning, e.g. in potato (invertase genes);
- yield benefits (e.g. RuBisCO genes), increasing catalytic activity and/or decreasing oxygenation activity and improved seed set in barley (e.g., homeodomain leucine zipper genes) ;
- improved biomass conversion for biofuels by lowering the lignin-content (caffeic acid O-methyltransferase gene).

2.4.8 Risk Relevant Issues

Comparable to the discussion provided for ODM (cf. Chapter 2.3.5) potential effects of SSN techniques may result on one hand from the mutation(s) introduced by SSNs.

On the other hand risk relevant issues are associated with the methods involved to introduce the molecular components of SSN systems into the targeted crop cells and the methods necessary to regenerate plants from mutated cells. For introduction of the SSN components usually GM technology is applied. Intermediate steps of the breeding process used to establish crops developed by SSN therefore require similar considerations than applications of GM technology. Thus the respective potential of these methods for unintended effects needs to be considered for identification of potential hazards.

Lastly SSN3 applications involve targeted insertion of additional genetic elements derived either from the genome of the targeted crop species (i.e. Cisgenes), recombinant genetic constructs involving genetic elements from the genome of the crop or related species (i.e. Intragenes), or recombinant genetic constructs containing elements from unrelated genomes (i.e. Transgenes). In such cases similar risk relevant issues as encountered with GM technology or Cisgenesis/Intragenesis (cf. chapter 2.5) will apply (EFSA 2012b).

With SSN applications known sequence elements (genes) are targeted for modification – either to introduce site specific mutations (e.g. by SSN1 and SSN2) or to insert additional sequences in a targeted way (e.g. by SSN3).

Possible mutations introduced can involve either small scale modifications, like point mutations of genes, leading to desired changes of gene product function(s), or larger scale modifications involving knock-out mutation which silence the expression of the targeted genes (loss of function).

At any rate the intended modifications introduced by SSNs will be stably inherited - comparable to mutations induced by ODM or conventional mutagenesis. The inheritance will follow a Mendelian pattern, if nuclear genes are targeted or similar to mitochondrial or plastid genetic elements, if such elements are targeted. On the contrary genetic constructs harboring transgenic SSN-genes or donor DNA-constructs are not intended to be present in the final breeding product. However they will be transiently present in targeted crop cells or additionally integrated into their genome during intermediate steps of SSN application.

As discussed for ODM above some kinds of unintended modifications through ODM are possible, e.g.

- Mutations introduced at off-target genomic locations sharing homologous sequences with the target site.
- Potential expression of fusion-proteins for some types of knock-out mutations.
- Unintended modification due to methods applied during transfection and regeneration.
- Unintended effects associated with GM modifications that are introduced in intermediate steps.
- Unintended effects, e.g. position effects, associated with Cisgenic/Intragenic/Transgenic constructs introduced with SSN3 techniques.

The respective risk issues thus need to be addressed by a comprehensive molecular characterisation, taking into account the experiences from risk assessment of GMOs.

Regarding risks associated by the engineered trait(s), again case specific considerations will have to be applied (see also Chapter 2.3.5 on ODM).

It can be anticipated that for some types of mutations introduced by SSNs, specifically mutations involving larger scale modifications, e.g. larger sized deletions or chromosomal rearrangements, the knowledge on the resulting effects will not be sufficient to conclude on the safety of the respective NPBT-crop without further assessment.

As noted by EFSA (2012b) the framework for assessment of health and environmental risks as developed for GM crops (cf. EFSA 2010 & 2011) is considered to be appropriate for crops developed by SSNs.

Initial applications of SSNs include the development of crops with important agronomic traits, e.g. HT-traits. As discussed for analogous ODM applications, important risk relevant issues that need to be considered are the (environmental) impacts of changes in agronomical management due to exploitation of these traits during crop cultivation.

2.5 Cisgenesis and Intragenesis

In principle for genetic transformation genes from all organisms, i.e. all plant and animal species as well as microorganisms may be used for insertion into a recipient plant. Trans-kingdom gene transfer as applied in genetic transformation has beside risk related debates also stirred ethical discussions. So concepts like cisgenesis and intragenesis have evolved which limit the source of genes used for gene transfer to those which are theoretically also available in traditional plant breeding.

The Concepts of Cis- and Intragenesis

In cisgenesis and intragenesis only the gene pool of the recipient species and/or of sexually compatible species is used as a source for the genetic constructs to be inserted. Sexually compatible species may be closely related species, like for instance different cultivars or related wild species.

Currently a lot of efforts are focused on the sequencing and assemblage of the structure of various relevant plant genomes and on specifying the function of the identified genes. Therefore an increasing number of isolated and well characterized genes are available for cisgenic and intragenic approaches.

Cisgenic and intragenic crop plants are generated by similar methods of gene transfer as used in transgenesis. Among other techniques predominantly *Agrobacterium*-mediated transformation and biolistic transformation are used to insert certain genetic constructs (or native genetic elements with a known function) at random sites into the recipient genome. While a cisgene is an exact copy of a natural gene (containing its native promotor and terminator), an intragene may comprise natural functional elements originating from different genes from the recipient species or sexually compatible species (see 2.5.1 and 2.5.2). In the latter case shuffling of the genetic material (e.g. genes, functional elements) is permitted.

It has to be noted however that in the scientific literature various terms are used to describe genetically modified organisms generated by using genes from the genome of the target species or sexually compatible organisms (for review see PRINS & KOK 2010, HOLME et al. 2013).

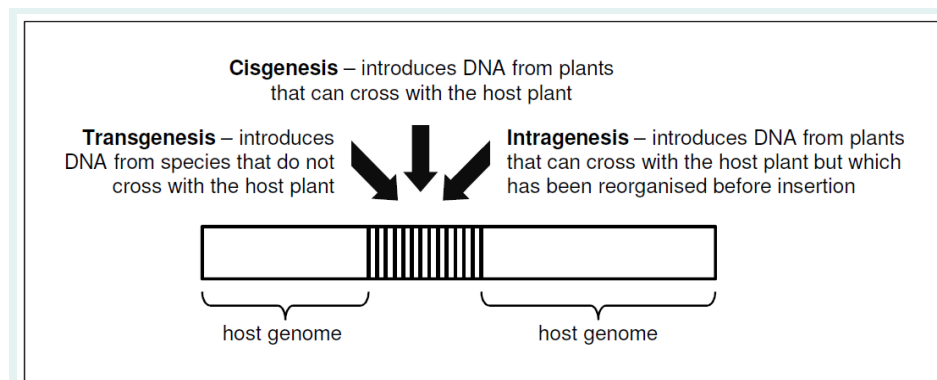


Figure 4: Schematic diagram of the three different forms of genetic transformation (transgenesis, cisgenesis and intragenesis) (adapted from ACRE 2013)

2.5.1 The Concept of Cisgenesis

The discussion of cisgenesis in this report is based on the definition provided by the EU Working Group on Novel Techniques (NTWG 2011), which addressed the issue whether applications of novel breeding techniques are subject to GMO regulations and whose definition is also referred to by EFSA (EFSA 2012a):

‘Cisgenesis is genetic modification of a recipient organism with a gene (cis-gene) from a crossable – sexually compatible – organism (same species or closely related species). The gene includes its introns and its flanking native promoter and terminator in the normal sense orientation.’

Cisgenic plants can harbour one or more cisgenes, but they do not contain any parts of transgenes or inserted foreign sequences. To construct cisgenic plants the same molecular biology techniques used for construction of transgenic organisms may be used. Genes must be isolated, cloned or synthesized and transferred back into a recipient where stably integrated and expressed.’

According to this concept one or more native genes of interest but no new DNA not belonging to the species’ natural gene pool is being introduced. The inserted genes as well as the associated introns and regulatory elements (e.g. promoter sequences) are used without any rearrangements and thus remain contiguous and unchanged.

As the concept of cisgenesis is rather broadly formulated and defined differently by various authors (PRINS & KOK 2010, HOLME et al. 2013), some details remain unclear. For instance the definition of the source of the cisgene may either comprise the natural gene pool or the plant-breeders gene pool which broadens continuously as new techniques become available. Another aspect is for instance the insertion of unwanted sequences, like vector backbone sequences. Regarding the latter the insertion of T-DNA borders is most often discussed (EFSA 2012a, NTWG 2011).

2.5.2 The Concept of Intragenesis

Again the discussion of intragenesis in this report is based on the definition provided by the EU Working Group on Novel Techniques (WGNT), whose definition is also referred to by EFSA (EFSA 2012a): 'Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism. Intragenesis involves the insertion of a re-organized, full or partial coding region of a gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species.'

In intragenesis like in cisgenesis only DNA from the target species itself or from cross-compatible species is being used as a source for generating genetic constructs for transformation. However in intragenesis these genetic elements may be rearranged *in vitro*. The inserted DNA thus can be a new combination of genetic elements resulting in a modified functional context as compared with the native genome. Moreover vectors may be constructed which use functionally identical (plant derived) P-DNA instead of T-DNA to avoid the accidental insertion of vector sequences (i.e. P-DNA concept). In addition the construction of vectors which entirely consist of functional equivalents of vector components derived from the recipient species or cross-compatible species, i.e. intragenic vector systems, is pursued (Conner et al. 2007). Thus the transformed plants do not contain any foreign DNA, even if border sequences from the vector are being inserted accidentally (see chapter 2.1.4 & 2.5.4).

2.5.3 Intended Modification

The intended modification depends on the regulatory elements and coding sequences contained in the recombinant construct which is integrated (see 2.5.5). In cisgenesis and intragenesis the same conventional transformation methods are used as for transgenesis to insert one or more specifically selected genes (gene knock-in). The intention is a stable integration of the inserted genes into the plant's genome to achieve a modification stably inherited across generations.

In principle with cisgenesis a similar genotype and phenotype could be achieved as with conventional breeding, but this is not likely for intragenesis (LUSSE & DAVIES 2013). However the advantage of cisgenesis, but also of intragenesis is that compared to conventional breeding no unwanted alleles derived from non-elite crossing partners are introduced in the progeny. These unwanted genetic elements, including alleles genetically linked to the gene of interest (i.e. located in the genomic neighbourhood of the desired trait/genes), are often responsible for severe losses in performance and quality. Therefore they have to be eliminated via repeated backcrossing, in which the progeny is crossed several times with the elite parental variety. Depending on the species concerned this is a time consuming and expensive task, especially for species with a long generation time (e.g. fruit trees), crops with complex genetics (e.g. polyploidy) and self-incompatible, vegetatively propagated crop plants (e.g. potato). Usually at least 5 steps of backcrossing are required to reduce the content of alleles derived from the non-elite crossing partner below 5% (AGES 2012).

Therefore cis- and intragenic approaches can substantially speed up the breeding process.

However in cisgenesis and intragenesis the selection process after modification is complicated, since selection markers commonly used during genetic modification of crops (e.g. herbicide or antibiotic resistance genes) cannot be used for these approaches. These selection markers are mostly genes of foreign/non-plant origin and thus must not be present in cisgenic or intragenic plants (SCHAART & VISSER 2009). Therefore if no traits useful for selection purposes are available from the plant breeders' gene pool, selection markers need to be removed from the final product (EFSA 2012a).

Cisgenic or intragenic constructs may also be designed to silence endogenous target genes by inducing RNA interference (RNAi). If stable loss-of-function phenotypes are induced by such an approach, they may resemble knock-out mutations obtained by mutation breeding (SCHAART & VISSER 2009).

2.5.4 Potential Unintended Effects of the Modification

Beside the introduction of the desired traits in cis- and intragenesis unintended alterations to the genome may occur, which might also make backcrossing steps necessary. These effects may result from the transformation technique applied, but may also be independent of the methodology due to natural processes and mechanisms at molecular level (see (WILSON et al. 2006)). In the following only those undesirable changes are described which result from the application of the transformation method. As these methods (e.g. direct gene transfer, *Agrobacterium*-mediated transformation) are the same as for transgenesis similar unintended effects may occur as in transgenesis.

Presence of non-plant sequences

When *Agrobacterium*-mediated transformation is used to transfer cis- or intragenes a minimal amount of foreign DNA (i.e. non-coding sequences from the vector) may be introduced: right and left border sequences flanking the transgene-DNA (T-DNA) or vector backbone sequences. The presence of vector backbone sequences is not accepted by definition in cis- and intragenic plants (JACOBSEN & SCHOUTEN, 2009). T-DNA border sequences are themselves non-coding and are unlikely to have phenotypic effects, but are of bacterial origin. Even though they are limited in number of nucleotides (e.g. up to 22-bp from the left border repeat), they may align with existing open reading frames (ORF) and thus can be translated into protein as part of a fusion protein.

In order to avoid even this small proportion of foreign DNA, specific vectors called intragenic vector systems have been developed in which only plant-derived transfer DNA (P-DNA) is used (CONNER et al. 2007, ROMMENS 2004). This is possible if due to the symbiotic relationship of the soil bacterium *Agrobacterium t.* and plants during evolution DNA sequences similar to the bacterial border sequences exist in a plant species genome. Using these sequences the insertion of bacterial DNA into the plant genome can be avoided. However plants derived by this method should be regarded as intragenic and not as cisgenic as the P-DNA vector has been constructed using reorganized plant-derived sequences (PRINS & KOK 2010).

Position effects

In principle the newly introduced genes can integrate at different locations in the genome (genetic loci). With standard transformation methods the insertion site cannot be predicted or controlled reliably. Thus so called 'position effects' are possible, which describe the fact that the expression patterns of identical genes differ depending on where in the plant genome they are inserted. However the random integration of genes can not only influence the expression of the inserted genes, but also have an effect on the expression of genes located around the insertion site in the recipient genome. For instance the cis- or intragene may be inserted into an existing gene which may lead to a mutation in the recipient genome at the insertion site (SCHAART & VISSER 2009). As a result the gene function in the recipient genome may be disrupted and unexpected phenotypic effects may be induced. Another possibility is that the cis- or intragene becomes part of an existing open reading frame (ORF) and a new, chimeric protein is produced (SCHAART & VISSER 2009).

Insufficient promotor sequence/promoter functionality

A promoter is a DNA sequence responsible for the expression of an associated gene and consists of various regulatory elements. Some of these elements may be located several kilo bases away from the transcriptional start site of the gene (PRINS & KOK 2010). In cisgenesis the native promoter is intended to be used for transformation. However this does not guarantee a similar expression pattern compared with the native gene, because regulatory elements connected to the cisgene in the native organism may be disconnected from it in the cisgenic organism. So if a cisgene is being isolated from a donor species without its full upstream promoter sequences this may lead to differences in gene expression (SCHAART & VISSER 2009). In intragenesis genes and promoter sequences may be rearranged within the intragene offering more options for the intentional alteration of expression levels and patterns. The resulting phenotype however may not be achievable by conventional breeding (SCHAART & VISSER 2009).

Multiple insertions

Multiple copies of the full-length cisgenic or intragenic construct or partial sequences thereof may be inserted in the genome at different sites. Furthermore direct or inverted repeats of the cisgenic or intragenic construct may be inserted at a single locus (SCHAART & VISSER 2009). Multiple insertions may have a significant effect on the quality and level of expression of the introduced gene.

2.5.5 Characteristics of the new traits from examples of plants, derived with Cis- or Intragenesis

Commercial development of cis- and intragenic plants are quite advanced in the EU, the US and New Zealand and have already reached the phase of field testing (LUSSEY et al. 2012, HOLME et al. 2013). In the EU field trials are being conducted with high-amylopectin potatoes, potatoes resistant to late-blight, scab resistant apples and barley with improved phytase activity (Tab. 4). With respect to the function of the traits used the focus lies on fungal resistance but also on compositional changes aiming at an enhanced product quality. In most cases the modifications aim at the overexpression of an existing gene or the expression of a new gene inserted from the plant breeder's gene pool (e.g. resistance alleles from closely related wild species). Besides gene silencing approaches were chosen for instance for potato and alfalfa (Tab. 4).

Theoretically also stacking of multiple genes would be possible, which would result in substantially modified plants. This approach would be particularly attractive for resistance traits as resistance determined by only one gene may more easily be overcome by pathogens than polygenic resistance. However making use of polygenic resistance traits requires that the respective endogenous resistance genes are characterised, isolated and ready for transformation which may often not be the case.

In addition it has to be noted that the examples presented in Table 4 describe work at different stages of development. So for some of the presented lines their cis- or intragenic nature may not yet have been sufficiently established (i.e. foreign DNA, like e.g. from marker genes or the vector backbone may still be present). However the true cis- or intragenic nature of the final product would have to be proven with respective molecular data presented in the notification procedure. If otherwise not only the intended modifications are contained in the cisgenic plant but also unintentionally foreign genes (e.g. from the vector backbone) are present, the plant is by definition transgenic.

Table 4: Overview on studies conducted using cis- or intragenic approaches

Trait/Characteristic of GM plant	Crop Plant	Transformation Concept	Approach/Type	Main Purpose of the Study	Reference
Resistance to <i>Phytophthora infestans</i>	Potato	cisgenesis	Expression	Jacobsen & Schouten 2009 present an example of a cisgenic approach (Testing of cisgenic plants with different <i>Rpi</i> genes or after stacking of <i>Rpi</i> genes in one clone)	HAVERKOORT et al. 2008 in JACOBSEN and SCHOUTEN, 2009
Downy mildew resistance (<i>Pseudoperonospora cubensis</i>)	Melon	Cisgenesis	Overexpression	Demonstration that susceptibility of a Melon cultivar is due to the downregulation of two genes and can be overcome by a cisgenic approach leading to overexpression of the respective genes	BENJAMIN et al., 2009
Increased phytase activity in grain	Barely	Cisgenesis	Overexpression of a phytase gene	Review article about the current status of development of cisgenic and intragenic crop plants	HOLME et al., 2012 ³
Different growth types	Poplar	Cisgenesis	Overexpression of various enzymes	Review article about the current status of development of cisgenic and intragenic crop plants	HAN et al 2011 ³
Enhanced fungal disease resistance	Grapevine	Cisgenesis	Overexpression of a pathogenesis-related protein	Review article about the current status of development of cisgenic and intragenic crop plants	DHEKNEY et al. 2011 ³
Scab resistance (HcrVf ²)	Apple	Cisgenesis and Intragenesis	Expression	Development of a cisgenic apple plant and of a intragenic apple plant	VANBLAERE et al., 2011, JOSHI et al 2011 ³
enhanced product quality ²	Potato	Intragenesis	Tuber specific silencing three genes	Recommendations for an updated regulatory approach to intragenic crops; incl. an example of the cisgenic approach in plant breeding	ROMMENS, 2007
Enhanced fungal disease (Gray mould) resistance	Strawberry	Intragenesis	Overexpression of the polygalacturonase gene	Review article about the current status of development of cisgenic and intragenic crop plants	SCHAART 2004 ³
Reduced lignin content	Alfalfa	Intragenesis	Silencing	Review article about the current status of development of cisgenic and intragenic crop plants	WEEKS et al. 2008 ³
Drought tolerance	Perennial ryegrass	Intragenesis	Overexpression	Review article about the current status of development of cisgenic and intragenic crop plants	BAJAJ et al. 2008 ³

¹ should lead to enhanced bioavailability of phosphate in barely feed; ² displayed in terms of increased starch level, reduced fry darkening, enhanced flavour and strongly reduced formation of acrylamide during heat processing; ³ in HOLME et al., 2013

2.5.6 Risk Relevant Issues

Characteristics which may cause potential adverse effects may either be the new genetic elements inserted or deletions and rearrangements of plant genomic DNA resulting from the genetic modification technique used (mainly *Agrobacterium*-mediated transformation). As the latter are the same as for transgenesis there is no difference regarding the possibility of unintended effects (EFSA 2012a, AGES 2012). Therefore a comprehensive molecular characterisation as required according to EFSA Guidance (EFSA 2010, EFSA 2011) is indispensable.

However with respect to the new gene products EFSA acknowledges that if the donor plant and the newly expressed proteins in cisgenic plants have a corresponding use and history of safe consumption as food and feed, depending on the case lesser amounts of event-specific data may be needed for the risk assessment. As the cisgene is *per definitionem* derived from the plant breeders' gene pool and remains unchanged compared to the donor plant the Panel concludes that similar hazards can be associated with cisgenic plants and conventionally bred plant (EFSA 2012). Regarding intragenesis however new combinations of genetic elements may arise and these may present novel traits with novel hazards (EFSA 2012).

In the following risk relevant issues are listed which are relevant for the evaluation of plants produced by cis- or intragenesis:

- Proteins may be expressed in cisgenic plants that have never been part of the human or animal diet (PRINS & KOK 2010).
- Increased expression of endogenous plant genes may affect the food and feed safety via altered biochemical properties.
- Cis- or intragenesis may lead to the disruption of existing ORFs or creation of new ones due to random insertion of the cis-or intragene in any part of the genome which in turn may lead to changes in the chemical composition of the plant
- Due to position effects the expression of the cis- or intragene may differ from expression of the endogenous gene in its natural genomic position
- Insertional mutagenesis (e.g. deletions, rearrangements) may occur at the insertion site of the cis-or intragene.
- As transformation methods usually are applied in cell cultures unintended changes in the plant genome may occur as a result of the in vitro culture called somaclonal variation.
- New combinations of native functional genetic elements are made in intragenesis which may lead to chimeric genes that do not exist in nature and whose expression levels and patterns thus do not correspond to that of the native gene (SCHAART & VISSER 2009).

Most of the above mentioned issues may have implications for the toxicity or allergenicity of the cis- or intragenic plant product. However changes in the composition may also display negative effects on non-target organisms. On the other hand as only genes from the plant breeders' gene pool are being used, the respective cisgenes are probably already be present in the population with which out-crossing may occur. Therefore risk associated with plant-to-plant gene transfer may not be as relevant as for transgenic plants. Overall the EFSA GMO Panel considers the Guidance for the risk assessment (EFSA 2010, EFSA

2011) applicable for the evaluation of cis- and intragenic plants and its derived food and feed products and sees no need for further development of the Guidance (EFSA 2012a).

2.6 Grafting on GM Rootstock (Transgrafting)

Grafting is a horticultural technique which has been practiced for centuries in particular in plant breeding of fruit species (e.g. apple, grapevine), but also of ornamental plants, like roses. In recent years also vegetables like tomato, cucumber, melon and eggplant are increasingly grown on rootstocks (COGEM 2006). Moreover grafting is widely used for the asexual propagation (i.e. cloning) of commercially grown cultivars. The combination of recombinant transformation techniques with the traditional practice of grafting opens up new possibilities for plant breeding.

The Concept of Transgrafting

Transgrafting describes the combination of traditional grafting practices with the genetic modification of crop plants. In grafting a bud-bearing part, the scion, is grafted on to a root-bearing part of another plant (e.g. a different variety of the same species or a different related species). Therefore the vascular tissues of both plant parts are placed in contact with each other and if vascular connection is established between them a chimeric plant is produced.

The plant providing the scion is selected for its stem, leave, bud or fruit characteristics and is fused with a rooted stem of another plant selected for instance for its pest or disease resistance or rooting characteristics. Advantages of grafting are for instance the possibility to induce dwarfing in fruit trees or to limit infection with soil borne diseases by using a resistant rootstock. In principle grafts between two different, but compatible species, called heterografting, are also possible. However grafting and thus transgrafting cannot be made use of in monocotyledonous crop plants like rice, maize and cereals.

In general the characteristics of both plant parts, the scion and the rootstock, may be improved by means of genetic modification and combined with GM or non-GM plant parts. Thus three different combinations are possible:

- Non-GM scions grafted onto GM rootstocks
- GM scions grafted onto non-GM rootstocks
- GM scions grafted onto GM rootstocks

The main focus in transgrafting is on the improvement of rootstocks by means of recombinant transformation techniques. In addition several examples of the grafting of a non-modified scion onto a GM rootstock have been reported (SCHAART & VISSER 2009), as an approach to improve the performance of the scion without introducing genetic modification to the harvested products (see also Table 5). Therefore this special case is of particular interest for the regulatory discussion and in the following focus is put on the combination of non-GM scions grafted on GM rootstocks.

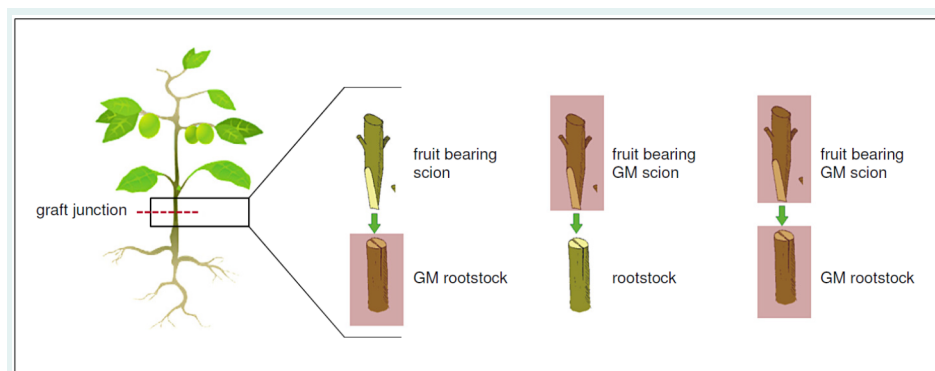


Figure 5: Schematic diagram of three different types of transgrafts resulting from the combination of grafting with recombinant transformation techniques (adapted from HAROLDSEN et al. 2012a).

2.6.1 Intended Modifications

Any kind of transgenic modification of the rootstock is possible with common transformation techniques. Most often the transfer of increased rooting ability traits or resistance traits is pursued (see Table 5). If the rootstock used for grafting has been genetically modified, according to current legislation the entire plant has to be considered genetically modified (NTWG 2011). Nevertheless its products (e.g. leaves, fruits) are not genetically modified as their DNA remains unchanged.

However it is known that upon grafting proteins and metabolites can be transported from the rootstock to the scion through the graft junction and vice versa. Thus effects on gene expression and phenotype in the respective other plant part (rootstock or scion) are possible. In combining grafting with recombinant transformation techniques (i.e. transgrafting) these mechanisms can deliberately be made use of. For instance the transmission of a genetically modified antimicrobial protein (Dutt et al. 2007 in SCHAART & VISSER 2009) and insecticidal Bt protein (WANG et al. 2012) from the GM rootstock to the scion has been demonstrated. In addition translocation of regulatory proteins (e.g. transcription factors), metabolites (plant hormones like e.g. auxins or cytokinins) or RNA in the graft can lead to epigenetic effects, e.g. on gene regulation in the scion.

Another possibility is to use genetically modified rootstocks to silence the expression of specific genes in the non-GM scion through RNA interference ('gene knockdown') (SCHAART & VISSER 2009) (see Table 5). It has been shown that the RNAi silencing signal in plants is mobile and can be transmitted through the graft (SCHAART & VISSER 2009). Depending on the target of the RNAi construct a silencing effect in the scion and in its fruits is possible. Usually the RNAi silencing aims at the modification of quality traits or flowering time (e.g. silencing of floral repressor genes in order to facilitate breeding). The RNAi construct is not integrated into the scion genome and therefore also neither contained in fruit products harvested from the non-GM scion nor sexually transmitted to the progeny (SCHAART & VISSER 2009).

2.6.2 Potential Unintended Effects of the Modification

As for the transformation of the rootstock recombinant transformation techniques are used, the same potential unintended effects may occur as have been described in chapter 2.1.4 (e.g. position effects, multiple insertions).

As has been highlighted above, mRNAs can move along the phloem long-distance translocation system. Potentially these mRNAs could be reverse transcribed into cDNA (e.g. by retroviruses) which could potentially be integrated into the genome. As discussed by Liu et al. horizontal gene transfer between the rootstock and the scion would be possible via this mechanism (LIU et al. 2010; ACRE 2013).

RNAi silencing of specific target genes can also influence DNA methylation patterns, resulting in epigenetic effects on gene regulation (e.g. gene silencing, upregulation of gene expression) (SCHAART & VISSER 2009). This change does not alter the sequence of the DNA, but modifies the chemical structure of the DNA bases (i.e. methylation of a DNA base). In certain cases the corresponding phenotype may be stably inherited by the next sexual generation. So the progeny is not genetically modified, even though as the result of the DNA methylation the altered phenotype is maintained in the following generations.

2.6.3 Characteristics of the new traits from examples of plants obtained with transgrafting

At present no commercial applications of plants grafted on GM rootstock are available (SCHAART & VISSER 2009). Most research activities in this field are related to the elucidation of the molecular mechanism underlying grafting in order to improve the grafting technique. A particular focus in research projects lies on the characterisation of the transmission of molecules through the graft junction aiming at a better understanding of the communication between the different parts of the grafted plant. However some field trials with GM rootstock grafted with non GM scions have already taken place in the EU with grape vine (ACRE 2013), apples (SMOLKA et al. 2010), peas, orange trees and citranges (LUSSEY et al. 2011). In China field trials have been conducted with poplar (WANG et al. 2012) and in Korea with watermelon (KIM et al. 2008).

Traditionally grafting is employed to improve disease resistance (in particular against soil-born fungi and bacteria) and growing aspects (e.g. rooting ability, nutrient and water acquisition) although the mechanisms are frequently unknown (HAROLDSEN et al. 2012b). Table 5 gives an overview on traits and crops plants which are involved in transgrafting experiments. Regarding the function of the traits used research activities mainly focus on virus resistance. However uncertainties remain regarding the movement of transgenic molecules to non-GM plant parts which cannot be demonstrated in all cases (AGES 2013).

The respective traits are stably integrated into the respective GM plant part (i.e. root or scion). If the respective trait is integrated in the scion's genome vegetative propagation as well as sexual transmission is possible. In case RNAi-based silencing is applied it may lead to changes in the methylation pattern of the genomic DNA. This can result in epigenetic effects on gene regulation, which may be maintained during clonal propagation or re-grafting (SCHAART & VISSER 2009).

It is evident that transgrafting activities are focused on perennial crops, especially fruit trees. An advantage of non-GM scions grafted on GM rootstocks would be that transmission of GM traits by pollen flow, which is an important issue in the risk assessment of GM crops, would not be an issue as the scions would not be GM. If this approach is found to be successful then different scion cultivars may be grafted onto one authorized GM rootstock. As a result the portfolio of potential applications of a certain GM rootstock would be wider and consequentially marketing opportunities and the potential return on investments would increase. Another reason for pursuing such approaches might also be the fact that the (fruit) products harvested from such transgrafts would not be genetically modified. This could facilitate on the one hand the risk assessment conducted for such products and on the other hand the marketing potential of authorized products, because these products might be met with higher levels of consumer acceptance.

Table 5: Overview on studies conducted with transgrafts and the various traits expressed in GM rootstocks (CGMMV = Cucumber green mottle mosaic virus, PNRSV = Prunus necrotic ringspot virus, GFLV = Grapevine fanleaf virus, n.a. = not assessed)

Trait/Characteristic of GM plant	Crop Plant	Results Concerning the Translocation of Molecules	Main Purpose of the Study	Reference
Insect-resistant rootstock and scions (Cry1Ac)	Poplar	Bt Protein moved bi-directionally in phloem sap through graft junction	understanding of transport of Bt protein within grafts	WANG et al., 2012**
Stimulation of rooting (<i>rolB</i>)	Apple	No translocation of <i>rolB</i> gene or its mRNA detected	Effects of transgenic rootstock on non-GM scions under natural conditions	SMOLKA et al., 2010 **
Crown Gall resistant GM rootstocks (expression of siRNA)	Walnut, Tomato	Transgenic DNA, protein and mRNA not detected in scion; siRNA mobilization not detected in walnut or tomato scions, but in walnut kernels	Translocation and mobility of transgenic DNA, protein, mRNA and siRNA	HAROLDSEN et al., 2012a*
Expression of RNA ¹	Apple	RNA-mediated gene silencing signals not graft transmissible	Examination of the possibility of graft-transmitted gene silencing of endogenous and transgenic gene sequences in apples	FLACHOWSKY et al., 2012*
Virus resistant rootstock (CGMMV)	Water-melon	n.a.	Effect on non-target organisms (aphids, thrips) in the greenhouse	HOONBOK Y. et al., 2006**
Virus resistant GM rootstock (tobamoviruses)	Tobacco	siRNA detected in non-GM scion and GM rootstock	Graft transmission of RNA silencing signals	ALI et al., 2013**
Virus resistant rootstock (PNRSV)	Cherry	n.a.	Transformation of rootstock (with a RNA interference vector)	SONG et al., 2013**
expression of a gene involved in meristem development ²	Pear, Tobacco	NACP mRNA transported between rootstock and scion in both directions	Transport of mRNA between rootstock and scion	ZHANG et al., 2013*
Virus resistant rootstock (GFLV)	Grape vine	n.a.	Potential promotion of the development of viable GFLV recombinants	VIGNE et al., 2004**
Virus resistant rootstock (CGMMV)	Water-melon	mRNA and protein were detected in GM rootstocks but not in scion	Molecular assessment of transgenic rootstock	YOUK et al., 2009**

¹ RNA used to silence a reporter gene expressed in transgenic scions; ² transcripts of *Pyrus-NACP* were followed in non-GM *Pyrus* grafts and GM tobacco grafts;

* research article; ** application-oriented article

2.6.4 Risk Relevant Issues

Regarding the regulatory status of transgrafts some scientists argue that if one part of the plant is genetically modified, the entire plant should be considered as genetically modified (SCHAART & VISSER 2009). Accordingly the EU Commission Working group concluded that the whole plant is captured by EU legislation on GMOs (NTWG 2011). At the same time the products derived from non-GM scions grafted on GM rootstock are not considered to fall under the scope of EU Directive 2001/18/EC, because they do not contain foreign DNA.

As transgrafting *per definitionem* involves the genetic modification of either the rootstock or the scion, unintended effects resulting from the application of the transformation method used (e.g. *Agrobacterium*-mediated transformation) are possible (see 2.5.4). It has to be noted that in general despite substantial experience with grafting the understanding on the molecular level of the influence non-GM rootstocks exert on scions is still rather limited (ALONI et al. 2010, Liu et al. 2010). However the combination of transgenesis with grafting techniques raises specific questions in particular regarding the transmission of gene products (e.g. proteins, RNAi constructs) through the graft union (for review see (HAROLDSEN et al. 2012b, ACRE 2013, AGES, 2013. Some of these issues are listed below and would need to be given special attention in the risk assessment of transgrafts:

- Potential effects of graft transmittable metabolites and compounds (dependent on their nature and their concentration) need to be addressed as they may have unintended effects on the scion and thus on non-target organisms:
 - The movement of transgenic proteins (e.g. Bt toxins, antimicrobial protein) in the phloem across the graft union has for instance been demonstrated in grafted poplar, grafted cotton and grafted grapevine (WANG et al. 2012, Rui et al. 2005 in WANG et al 2012. DUTT et al 2007 in SCHAART & VISSER 2009).
 - Proteins like transcription factors involved in gene regulation as well as plant hormones (e.g. auxins, cytokenins) may be transported through the graft union and may affect the physiology of the scion (SCHAART & VISSER 2009).
- If RNAi-mediated silencing is used depending on the target of the RNAi construct the silencing signal may also be effective in the scion (e.g. silencing of floral repressor genes) as translocation of these small non-coding RNAs in the plant is possible. Depending on the mode of action of the RNAi signal this effect may either be sexual transmittable or not. For instance if RNA-directed DNA methylation is induced, this may lead to either silencing or up-regulation of gene expression and the resulting phenotype may be stably inherited by the next sexual generation (SCHAART & VISSER 2009). This however is relevant for risk assessment as potential effects resulting from plant-to-plant gene flow need to be addressed.
- Although it is considered extremely unlikely that genomic or organelle DNA would be mobile over long distances, exchange of genetic material may occur near the graft junction and has been demonstrated for plastid DNA in tobacco grafts (STEGEMANN & BOCK 2009).
- Depending on the species adventitious shoots (e.g. suckers) may develop on the GM rootstock and may produce leaves and fruits that are GM. This possibility has to be taken into account as for instance it significantly changes the

exposure of non-target organisms to transgenic proteins or the possibility for plant-to-plant gene flow.

- Depending on the nature of the genetic modification, in particular if dsRNA is involved (e.g. RNAi), the interaction of GM–rootstock with the soil environment may have an impact on soil organisms like e.g. nematodes, which are capable of directly taking up dsRNA from the environment (AGES 2013)

2.7 Techniques to support breeding (TSBs)

A number of different NPBTs are addressed in this chapter all of which are designed to facilitate traditional plant breeding approaches and address inherent difficulties encountered in conventional plant breeding. A number of these difficulties cannot easily be addressed by other means of conventional breeding. E.g. the long juvenile phase of certain long-living crops, e.g. trees, can result in cross-breeding schemes that are extremely time consuming. Other difficulties are associated with the production of seed material for cultivation of specific hybrid elite varieties: On one hand the production of hybrid seed material in self-fertile crop species can be infeasible or be associated with high costs and efforts. On the other hand specific selected elite hybrid plants showing a desirable combination of traits valuable for agricultural cultivation cannot be reliably reproduced if appropriate parental breeding lines are not available.

The Concepts of TSBs

In the following three approaches are described where NPBTs have been developed to support cross-breeding:

- **Reverse breeding (RB)**

RB is applied to facilitate the reproduction of specific hybrid elite lines in case appropriate homozygous parental lines are not available.

To establish such parental lines a specific heterozygous (hybrid) elite line is chosen for its phenotypic characteristics. This hybrid plant is then modified to suppress meiotic recombination and haploid gametes are converted into double-haploid plants. These plants are screened to identify a pair that would on the one hand reconstitute the original heterozygous plant and secondly would not carry the modification. This pair of homozygous lines is used subsequently to produce seed material for cultivation of the original hybrid elite line (c.f. LUSSEY et al. 2012).

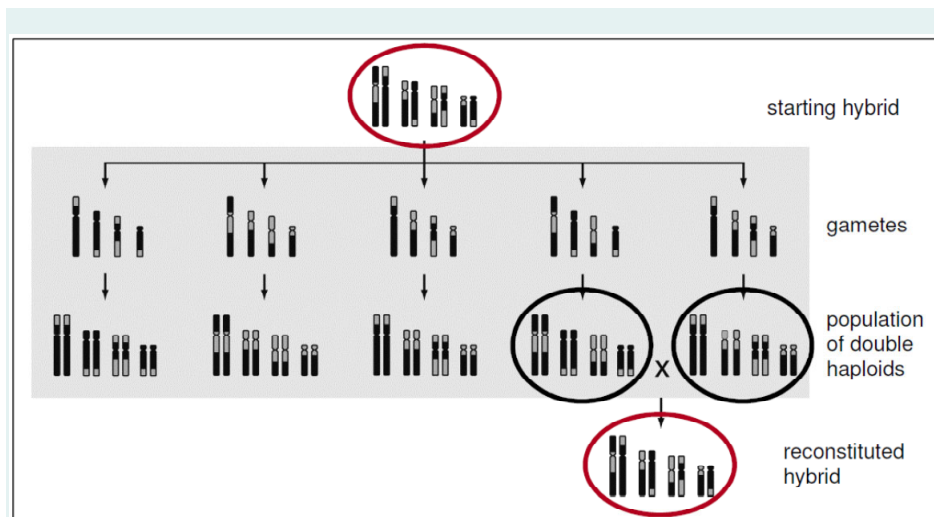


Figure 6: Schematic representation of the steps involved in reverse breeding (adapted from DIRKS et al. 2009)

● Seed production technology (SPT):

SPT is used for streamlined production of hybrid seed material involving male-sterile breeding lines (c.f. VOGEL 2012).

With SPT parental male-sterile breeding lines for the production of hybrid seeds can be maintained and reproduced. Male-sterile lines usually cannot be reproduced as inbred lines. In SPT however such male-sterile breeding lines are bred with isogenic maintainer lines which carry GM modifications to reconstitute their fertility and a marker gene. Offspring seed which does not contain the GM modifications is then detected by absence of the marker gene. Plants grown from such seed are again showing a male-sterile phenotype and thus can be used for production of hybrid seed material without labour intense and costly manipulation (e.g. detassling of all maize plants prior to fertilisation).

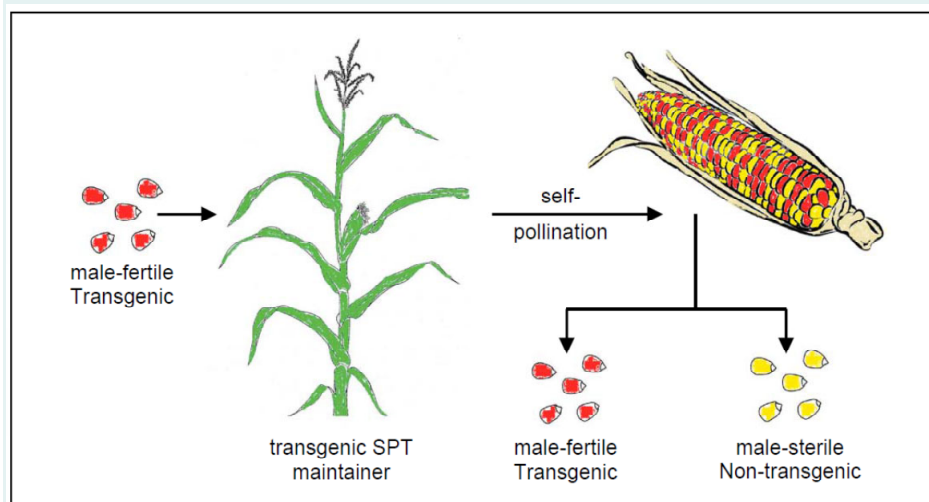


Figure 7: Schematic representation of involvement of a transgenic maintainer line in production of seed material of a male-sterile parent line for hybrid seed production. (adapted from Pioneer 2014):

- **Accelerated breeding (AB):**

AB is applied for the induction of early flowering in breeding intermediates to significantly shorten the juvenile phase of crop plants, particularly of long-living crops, e.g. trees. AB thus allows to speed-up cross-breeding approaches in crop development (e.g. SCHAART & VISSER 2009).

Typically with all these different TSBs a biotechnological modification is introduced as a means to facilitate an intermediate step in breeding rather than an ends, i.e. the production of a modified breeding product. Usually the application of these NPBTs involves introduction of transgenes by GM technology during the breeding process into intermediate breeding products or maintainer lines, which facilitate the multiplication of male-sterile parental plants for hybrid production. Typically the products of the breeding process are plants which are selected to not contain the GM modifications any more, but do exhibit the intended traits.

2.7.1 Reverse breeding

RB is actually a combination of different techniques which are applied in a sequential manner to generate homozygous lines parental lines which recreate a desired heterozygous genotype. The resulting breeding products are in essence identical to the initial elite hybrid crop, which is the starting point for the breeding process (e.g. NTGW 2011).

The steps involved in RB can be listed as follows (AGES 2013):

1. An elite heterozygous line is selected for its phenotypic characteristics.
2. Meiotic recombination is suppressed (e.g. through RNA interference, RNAi).
3. Gamete cells that do not contain the transgene are regenerated into homozygous, double haploid plants.
4. Parental lines are selected which together will reconstitute the initial heterozygous phenotype – only non-transgenic plants are selected.
5. The desired heterozygous genotype is obtained via crossing of the selected parental lines, resulting in final heterozygous plants being non-transgenic.

The different techniques applied are then:

At Step 2:

A number of techniques may be applied resulting in silencing of meiotic recombination during sexual reproduction. Usually this is achieved by introducing GM modifications, which lead to silencing of genes required to initiate meiotic recombination events (SCHAART & VISSER 2009). In a proof of concept experiment with *Arabidopsis thaliana* a GM based dominant RNAi approach was used to silence the DMC1 gene to suppress crossover recombination (WIJNKER et al. 2012). However other techniques were discussed for achieving this objective (DIRKS et al. 2009), among them virus induced gene silencing and grafting on GM rootstock to deliver silencing construct, introduction of dominant-negative alleles and use of chemical inhibitors.

Application of GM methods is usually associated with propagation of individual cells in cell culture and/or with phases of in vitro tissue culture.

At Step 3:

During this step microspore propagation and double-haploid techniques may be applied to convert haploid gametes into diploid plants which are homozygous for chromosomes derived from the initial hybrid (c.f. VOGEL 2012). Other approaches resulting in balanced double-haploid offspring may be used depending on the plant species (WIJNKER et al. 2012).

Again cell/tissue culture methods may be required at this step.

In addition to reconstitution of heterozygous genotypes RB can also be used to create chromosome-substitution lines which are valuable in many breeding applications, such as trait mapping, the study of epistatic interactions and targeted inbreeding (WIJNKER et al. 2012, see also VOGEL 2012).

2.7.2 Seed production technology

SPT is addressing a disadvantage with the use of male-sterile breeding lines namely the difficulty to propagate the male-sterile line as an inbred line. To circumvent this disadvantage a so-called maintainer line was developed which allows easy production of inbred seeds of the male-sterile line, which can in turn be used as a female partner for the production of commercial hybrid seed material (see VOGEL 2012).

The transgenic maintainer line contains a cassette of genes that restores fertility and prevents functional transgenic pollen from being produced. The cassette also includes a colour marker gene (e.g. dsRed) that makes appear pink under ultraviolet light due to fluorescence of the transgenic protein (see WALTZ 2012, VOGEL 2012). Due to the included selection marker transgenic and non-transgenic seeds can be automatically colour sorted and segregated. This should ensure that only transgenic maintainer plants are used for propagation of the male-sterile line, whereas only non-transgenic plants of the male-sterile line are used for commercial seed production.

2.7.3 Accelerated breeding

With plant species with longer generation times, e.g. trees, conventional breeding approaches are usually very time consuming. For example with fruit trees like apple period of greater than 50 years may be needed to obtain a new apple cultivar with marketable quality which is expressing a trait originally present in a wild apple variety (c.f. FLACHOWSKY et al. 2011). AB approaches may be used to significantly reduce this time by induction of early flowering and thus shortening the juvenile phase (see SCHAART & VISSER 2011; WALTZ 2012). Results from studies of the induction of flowering in model plants e.g. Arabidopsis were used to devise different strategies to manipulate the length of the juvenile phase in crop plants, among them perennial plants like trees as well as annual crops (see VOGEL 2012).

One approach is to silence vegetative maintenance factors, e.g. TFL1, using transgenic RNAi constructs. Silencing of the TFL1-gene was shown to result in early flowering (see SCHAART & VISSER 2009). Other approaches rely on GM modification to overexpress flowering initiation factors, e.g. BpMADS4 or pFT1, in apple or plum trees to achieve the same effect (FLACHOWSKY et al. 2011, LE ROUX et al. 2012, Srinivasan et al. 2012). These early flowering GM plants can

then be used in breeding programs until the breeding objective is achieved, e.g. development of quality traits or disease resistance. In a final breeding step the GM construct is segregated and non-GM plant lines are selected as a final breeding product.

As reviewed by VOGEL (2012) alternative approaches other than transgenesis for silencing of endogenous plant genes to induce early flowering are discussed, e.g. virus induced silencing or transgrafting on GM rootstocks producing siRNAs that are transmitted into the non GM scion.

2.7.4 Intended Modifications

As regards TSBs the intended modifications introduced by the used NPBTs into intermediary breeding lines have to be distinguished from the intended modifications of crop traits according to the targeted breeding objective.

As a direct result the application of the respective NPBTs will give rise to GM-intermediates harbouring transgenic constructs which result in different traits:

- With RB applications primary modification are targeted to changes to the reproductive cell division system, i.e. inhibition of meiotic recombination. Such modifications severely impact the balanced distribution of parental chromosomes during meiosis and thus the production of viable gametes without chromosomal aberrations, i.e. balanced gametes (WIJNKER et al. 2012). The frequency of unbalanced gametes to occur is different for different crop species and is increasing with the number of chromosomes.
- The primary modification introduced for SPT also has effects on the reproductive system and thus the fertility of plants harbouring the modification. Additionally transgenic fluorescent marker genes are introduced into the maintainer line according to the approach described above (see also USDA 2011).
- AB is based on primary GM modifications resulting in changes of plant growth and development, as well as changes in morphology. E.g. AB plum intermediates displayed pleiotropic phenotypes atypical for plum including shrub-type, bushy growth habit, weepy branches and changed flower architecture (Srinivasan et al. 2012; WALTZ 2012).

The genomic modifications introduced by the NPBT are in most cases similar to those seen with other GM modifications and the introduced transgenes are resulting in significant phenotypic changes in important features of the crop biology, that are quite easy to spot. However these modifications will not intentionally be present in the final breeding products.

Typically the intended changes of crop traits in the respective breeding products will be independent from changes introduced by the NPBTs itself.

The products of RB are not considered to contain any intended genomic modifications that are not present in the hybrid crop selected as a starting point for RB. Similarly the products of SPT are only meant to inherit traits present in the male-sterile female and the other elite parent line used for hybrid production. The breeding products from AB applications would not be expected to harbour new traits that could not be also introduced by conventional breeding. However it has to be noted that the range of traits subject to such breeding approaches is reasonably broad, particularly if breeding is supported by MAS. Quantitative and qualitative traits from distantly plant relatives (including non-crop species) may be introduced. As stated by VOGEL (2012) traits can also be introduced from

plants which are not fully sexually compatible via embryo rescue or by means of bridge-crosses into compatible breeding partners. In case the knowledge about the effects of such traits is limited, they may carry an unknown potential for adverse impacts.

2.7.5 Potential Unintended Effects of the Modifications

The concept of all TSBs is that the GM modifications introduced to facilitate the breeding approach are segregated out to generate non-GM lines as breeding products. However a number of unintended effects should be taken into consideration.

These unintended effects on the one hand can be associated with unintended modifications due to the application of GM technology. Undetected secondary insertions of the transgenic constructs may be retained during segregation and approaches based on the silencing of target genes by RNAi and may also initiate the RNA-directed DNA methylation of the transcribed region, which can change the expression of the target genes. Since sometimes the changed methylation-patterns are transmitted to the offspring, changed phenotypes due to epigenetic regulation may be preserved in subsequent generations (SCHAART & VISSER 2009). Depending on the homology of the target genes with other endogenous genes also regulation of such genes may be affected unintentionally.

All procedures include phases when crop cells are propagated in tissue culture. As with other breeding techniques, cell propagation in tissue culture may induce unintended changes in the crop genome by somaclonal variation (cf. EFSA 2012a & 2012b). In vitro propagation of gametes (e.g. microspore culture as a step in the generation of double haploid plants) may lead to gametoclonal variation (VOGEL 2012). Both processes may generate genomic as well as epigenetic effects (see references in VOGEL 2012). The conditions of culture influence type and extent of effects.

2.7.6 Characteristics of the new traits from examples of plants obtained with TSBs

As regards RB the “Proof of Concept” for the approach was demonstrated by Wijnker and colleagues (2012) using Arabidopsis as an experimental model which is not an important agricultural crop itself. LUSSEY & DAVIES (2013) note that several patents on the technique have been filed by a Dutch company and crops developed by this technique are still in the research phase.

SPT according to the outline presented above was developed by Pioneer Hybrid company (cf. PIONEER 2014). The company recently started to market commercial maize hybrids produced with SPT in the U.S. Rice hybrids produced using the SPT process are still in an early phase of development (Proof of Concept) according to the developer (PIONEER 2014).

AB is still in research & development according to SCHAART & VISSER (2009). The concept is applied to a number of crop species, including fruit trees like apple (LE ROUX et al. 2012, FLACHOWSKY et al. 2011) and plum (Srinivasan et al. 2012). However the range is also including other trees species (citrus and pear trees) and annual plants as reviewed recently (see VOGEL 2012).

2.7.7 Risk Relevant Issues

Risk issues are primarily connected with the traits that are targeted by the specific breeding approaches rather than with modifications by the NPBT-techniques applied in RB, SPT and AB.

Breeding objectives for AB may involve genes from the secondary and tertiary gene pool of a crop plant, including alleles which have not been used earlier in crop varieties for food and feed production. Alleles influencing quantitative target traits or introducing disease resistance may have pleiotropic effects in the final breeding product. Experience with these effects may be limited (cf. VOGEL 2012).

Additional traits genetically linked to the target trait may be introduced (linkage drag). If linkage is tight such unintended traits may still be present in the final product after cross-breeding for a number of generations.

Some target traits e.g. herbicide tolerance, can be connected to indirect ecological effects also encountered with GM crops exhibiting similar traits. Thus (environmental) impacts of changes in agronomical management due to exploitation of these traits during crop cultivation should likewise be considered (cf. EFSA 2010).

As regards the initial GM modifications due to NPBTs a thorough characterisation of the final products of RB and AB is needed to exclude the unexpected presence of GM modifications. As a result of these initial modifications the breeding products may also exhibit phenotypes transmitted as inheritable epigenetic traits. The final breeding products should be assessed for traits expected for the initial modifications, e.g. meiotic aberrations, early flowering). Also unintended adverse effects, e.g. transmittable off-target regulatory effects need to be considered. This requires a thorough phenotypic assessment of the breeding product in case molecular evidence cannot exclude off-target effects.

Maintainer lines for SPT need to be grown in containment, or risk assessed according to GM regulation (EFSA 2010 & 2011). The absence of transgenic traits contained in the maintainer lines needs to be confirmed by appropriate monitoring in the male-sterile offspring used for production of hybrid seed material for commercialisation.

For a comprehensive evaluation the impact of somaclonal or gametoclonal variation in the final breeding products need to be considered, if methods including GM techniques are used, which depend on in vitro cultivation steps (VOGEL 2012). Somaclonal or gametoclonal variation may result in random genetic or epigenetic modifications, including chromosomal aberrations, deletions, mutations affecting sequence and expression of specific and induction of transposition events (see VOGEL 2012). Such effects are not specific for the NPBTs in question and will also occur if the respective in vitro cultivation methods are used for conventional breeding and GM modification.

However certain conditions of in vitro cultivation are known to favour the generation of such effects (cf. BAIRU et al. 2011). It needs to be considered if the methods applied during a certain NPBT approach specifically favour generation of somaclonal or gametoclonal variation.

2.8 Agroinfiltration

Agroinfiltration techniques exploit a naturally occurring mechanism by which certain soil bacteria, particularly *Agrobacterium tumefaciens*, can infect host plants and introduce episomal genetic material, i.e. Ti-plasmids harboured in the bacteria, into plant cells. Genes contained within the introduced Ti-elements can be expressed in the plant cells and in nature modify the metabolism of infected cells to support *Agrobacteria* growth and development. This mechanism is also used as the most common method in transgenesis to transform plant cells with recombinant DNA (c.f. LUSSE et al. 2013, SCHAART & VISSER 2009). However the Agroinfiltration approach is different since with most applications a stable integration of the introduced recombinant DNA into the genome of the infected host plant is not intended (see AGES 2012, VOGEL 2012)

The Concept of Agroinfiltration

In agroinfiltration applications tissues of the target plant are infiltrated with a liquid suspension of *Agrobacterium* cells. The T-DNA of these *Agrobacteria* is genetically modified to contain recombinant constructs designed for expression in host plant cells (OECD 2014). Localised inoculation of somatic or generative plant tissues with such *Agrobacteria* may be achieved by infiltration of such plant parts (leaves, roots, floral tissues) by means of infiltration with syringes or vacuum suction, spraying plant parts with the *Agrobacterium* solution or dipping the targeted plant parts (roots, floral buds) into the suspension (see VOGEL 2012). As a result high numbers of recombinant constructs are transmitted into the nuclei of the target plant cells and the transgenes contained in these constructs are expressed in high quantities in the infiltrated plant tissues.

Agroinfiltration on the one hand provides a quick and simple method for high-level expression of transgenes in specific tissues in vivo or in explanted tissues (SCHAART & VISSER 2009). On the other hand the introduced recombinant constructs can be designed to achieve silencing of endogenous plant genes via the RNAi-pathway (c.f. SCHAART & VISSER 2009, VOGEL 2012). Several types of applications of agroinfiltration can be distinguished (LUSSE et al. 2011). The differences are due to the non-replicative or replicative nature of the introduced constructs, i.e. the ability of the recombinant T-DNA to be replicated in the host cells or not, and to the plant tissues (i.e. vegetative or generative tissues) which are infected:

- **Agroinfiltration sensu strictu:**

In such approaches non-germline tissue (typically leaf tissue) is infiltrated with non-replicative constructs in order to obtain localised expression of the respective transgenes in the infiltrated area (LUSSE et al. 2011). In result a local and transient expression of the introduced transgenes is facilitated or plant genes are silenced in the infiltrated tissues following expression of transgenic constructs leading to RNAi (SCHAART & VISSER 2009, Vogel 2012).

- **Agroinfection:**

For this type of applications replication-competent viral vector sequences are included in the respective T-DNA constructs used to infiltrate non-

germline tissues of the target plant. The contained viral vector construct is replicated in the infiltrated cells and is spread throughout the whole plant or in certain plant parts dependent on the infection properties of the virus (LUSSEr et al. 2011). As with agroinfiltration *sensu strictu* transient expression of transgenic proteins or the silencing of endogenous plant genes can be achieved (VOGEL 2012).

- **“Floral dip”:**

In this type of approach germline tissues (typically flowers) are exposed to a suspension of *Agrobacterium* carrying a transgenic T-DNA construct. The construct is expected to be transferred into female gametes and to eventually result in the genetic modification of embryos that can be selected at the germination stage. Thus with floral dip applications stably transformed plants may be obtained, comparable with GM transformation methods (LUSSEr et al. 2011).

Agroinfiltration methods may be used for a number of different applications:

Agroinfiltration *sensu strictu* and agroinfection are targeted to transient expression of the introduced transgenic elements or to modification of the expression of endogenous crop plant genes, typically by silencing them (see VOGEL 2012). As reviewed by AGES (2012) and VOGEL (2012) such approaches can be useful as tools to address several objectives relevant for plant breeding. They may be used e.g.

- to study gene functions in crop plants as a research tool for rapid functional gene analysis,
- to facilitate the screening and selection of crop plants with valuable characteristics, e.g. disease and stress resistance traits,
- for the assessment of the functions and effects of genes which may be used as potential transgenes in GM crops subsequently (LECKIE & STEWART 2011,
- for high-level expression of commercial interesting transgenic proteins in plants (or plant tissues), e.g. for the production of Plant-made Pharmaceuticals (PMP) in molecular farming approaches (reviewed by AGES 2012 and VOGEL 2012), and
- as a potential tool to deliver the primary modifications to crops necessary for NPBT-applications like RdDM, reverse breeding and nuclease-mediated site-directed mutagenesis.

Whereas the above approaches are not targeted to generate stably transformed GM lines, “floral dip” applications are used as a streamlined tool to achieve transgenesis in plants, particularly in the experimental model *Arabidopsis* (c.f. AGES 2012).

2.8.1 Intended Modifications

With agroinfiltration *sensu strictu* and agroinfection the infiltrated plant or plant parts are the target of interest and not any (GM) modified offspring plants (LUSSEr et al. 2011).

After infiltration the *Agrobacteria* transfer significant numbers of transgenic T-DNA molecules into the target plant cells, which are initially present as extrachromosomal genetic elements in these cells. Rapid transient expression of

the contained expression cassettes contained in the transgenic T-DNA is then initiated and persists for short times, i.e. up to 12 days as reviewed by AGES (2012). Dependent on the introduced transgenic sequences the expression products may be transgenic proteins or functional RNAs, e.g. double-stranded RNAs which are able to induce silencing of complementary endogenous plant genes (SCHAART & VISSER 2009).

Effects are restricted to infiltrated cells/tissues, typically to the somatic cells/tissues treated with *Agrobacteria*.

During applications of agroinfection replication competent recombinant viral sequences are transferred into somatic crop plant cells with the transgenic T-DNA originating from the used *Agrobacterium* strain. In the plant cells on the one hand replication of the viral vector sequences takes place and the recombinant vector is spread in the plant. On the other hand transgenes contained in expression cassettes in the viral vector are transiently expressed in the infected plant cells. Spread of viral infection and duration of the expression of recombinant products is dependent on the characteristics of target crop and the used viral sequences; however a timeframe of some 2 to 16 weeks is indicated in VOGEL (2012) for effects of virus induced gene silencing.

If the viral vector persists in the infected plant and generative cells are infected the transgenic construct can be transmitted to offspring plants.

Persistent genetic modification and/or stable epigenetic modifications of infiltrated plant tissues are only generated by specific applications of the technique (c.f. SCHAART & VISSER 2009, VOGEL 2012):

- Floral dip applications are aimed to produce GM crop plants as a primary objective by modification of generative cells.
- Agroinfiltration may be used to express transgenes which initiate changes in the system of epigenetic gene regulation, e.g. by RdDM, or lead to targeted mutagenesis by nuclease-mediated site-directed mutagenesis. Such changes are only heritable if generative cells/tissues are affected (SCHAART & VISSER 2009).

2.8.2 Potential Unintended Effects of the Modifications

Since floral dip applications are targeted to introduce transgenic modifications, comparable unintended effects need to be considered as for other transgenic, cisgenic and intragenic crops, including effects due to presence of non-plant sequences, positional effects on the expression of inserted genes as well as on expression of endogenous genes located at or around the insertion site, effects of multiple (partial) insertions and effects due to instability (see also chapter 2.5.4.).

For the other applications integration of the T-DNA elements transferred by the used *Agrobacteria* is not intended, however it cannot be excluded (LUSSETER et al. 2011, NTWG 2011, SCHAART & VISSER 2009). *Agrobacteria* may spread throughout the infiltrated plant and integration events may thus also occur in somatic or generative cells selected for further propagation (see ref. in VOGEL 2012, SCHAART & VISSER 2009). The same issue is relevant for propagated material which was infected with recombinant virus sequences during agroinfection.

If applications involve the silencing of target crop genes by RNAi, unexpected effects due to inheritable epigenetic effects on gene regulation (on the target gene as well as on related non-target genes) may result

Again somaclonal variation due to in vitro culture steps – e.g. cultivation and regeneration of explanted somatic cells or in vitro methods applied to remove transgenic virus sequences from infected cells – may happen, if the implemented approaches involve such propagation steps.

2.8.3 Characteristics of the new traits from examples of plants obtained with Agroinfiltration

Agroinfiltration *sensu strictu* and agroinfection were mostly used for research in model plants, including *Arabidopsis* and tobacco. In particular, agroinfiltration was often used in functional gene assays, elucidation of plant-pathogen interactions or assessing the functionality of regulatory elements (LUSSETER et al 2011).

Other applications were aimed to overexpress high value recombinant proteins, in infiltrated plant parts for production of recombinant plant made pharmaceuticals, e.g. vaccines, antibodies and blood proteins for use in humans as well as animal therapeutics (see AGES 2012, LUSSETER et al. 2011 for references).

Additionally agroinfiltration and agroinfection were used for screening of pathogenicity factors and disease-resistance in crop plants like potato (Bhaskar et al. 2009) as well as for assessment of resistance factors ahead of further use in the construction of respective GM crops (LECKIE & STEWART 2011).

2.8.4 Risk relevant issues

As discussed above risk issues for application of floral dip would resemble the ones considered in a case-specific manner for applications of transgenesis (EFSA 2010 & 2011) and for cisgenesis/intragenesis (EFSA 2012a, AGES 2012).

If agroinfiltration *sensu strictu* and agroinfection are applied for selection purposes only, (genetic) modifications are not intended in any target crop material which is selected to be further propagated.

However adverse effects need to be considered which are caused by unintended integration of transgenic sequences due to spread of *Agrobacteria* or recombinant virus sequences in the infiltrated/infected plant (LUSSETER et al. 2011, SCHAART & VISSER 2009). Therefore the absence of modifications needs to be demonstrated in material which is used for further breeding. Relevant approaches to assess presence/absence of transgenic sequences have been established for the risk assessment of GM crops (EFSA 2010 & 2011).

In case agroinfiltration was used for gene-silencing, it needs to be considered whether the silencing effect occasionally is still present in the non-GM offspring generated by vegetative or sexual propagation. This is particularly relevant for use of agroinfiltration for induction of RNAi-mediated silencing by RdDM, since the changes to epigenetic regulation can be stably inherited by the next sexual plant generation (SCHAART & VISSER 2009). Therefore changes in the expression of the target genes as well as other likely-affected non-target genes need to be evaluated.

Furthermore the unintended release of transgenic *Agrobacterium* strains into the environment can result in adverse effects. The transgenic *Agrobacterium* may survive in soil and transfer transgenes either to other plants or via horizontal gene transfer to other microorganisms (SCHAART & VISSER 2009). For analogous reasons the release of transgenic plant viruses from agroinfected material is a concern.

Therefore any plant materials including seeds originating from agroinfiltration and agroinfection applications need to be tested rigorously for presence of transgenic *Agrobacteria*, transgenic virus and plasmid sequences and presence of T-DNA constructs (VOGEL 2012)

3 CONSIDERATIONS FOR THE RISK ASSESSMENT OF NPBT-CROPS

In Europe as well in many other countries worldwide crops obtained with GM techniques are subject to a mandatory notification procedure and have to undergo a rigorous risk assessment (RA). At the same time plant varieties obtained via conventional breeding methods (i.e. selection breeding, cross-breeding or mutation breeding) can be marketed without specific evaluation except testing for distinctness, uniformity and stability (DUS testing) obligatory for variety registration in Europe. With the advent of new plant breeding techniques the question arises how NPBT crops may be integrated into the current regulatory systems and a framework may be established for an appropriate assessment of the potential risks which might be associated with their application.

For the time being for NPBT plants the classification of the respective technique as producing/resulting in GMOs or not is decisive in determining their regulatory status (see NTGW 2011). However the question is pending whether the current definition of a GMO is adequate for deciding on the regulatory status of certain NPBT plants which are currently available or will be developed in the future. This is regarded to be a policy decision, however might be influenced also by safety considerations.

An illustrative example for the difficulties associated with such decisions is the development of HT crops by different approaches, e.g. GM technology, application of NPBTs or conventional breeding. These approaches are either subject to regulation (GM), or they are not (conventional breeding) or might not (NPBT) be subject to current regulations:

A number of existing traits conferring resistance to broadband herbicides have been introduced into crops by means of genetic modification. GM-mediated glyphosate resistance and glufosinate resistance traits are the most common and best known traits, but the portfolio includes other HT traits as well, e.g. resistance to herbicides like ALS-inhibitors (e.g. Imazamox). The respective GM plant had to undergo a mandatory risk assessment. However phenotypically similar resistance traits (to ALS-inhibitors) were also be introduced by other means than genetic modification. On the one hand conventional breeding based on chemical mutagenesis was used to produce crops resistant to Imazamox herbicides (GELINSKY 2013). On the other hand a similar trait was developed by oligo-directed mutagenesis (ODM) based on the rapid trait development system (RTDS) developed by the US breeding company CIBUS (CIBUS 2013). Resistance to Imazamox has so far been introduced in many crops like e.g. sunflower, wheat, rice and oilseed rape.

From an environmental and agronomic point of view the main problems associated with the Clearfield System however are basically the same as for other herbicide resistant crop plants particularly other ALS-inhibitor traits: problems associated to volunteers in subsequent crops, which can no longer be controlled with the respective herbicide, increased development of resistance in weeds species through the increased use of the respective herbicide in the crop rotation and other indirect effects resulting from the changed agricultural management system (e.g. increased use, use of herbicide mixtures to maintain control).

The NTWG considers ODM to be captured by Annex IB of EU Directive 2001/18/EC and thus to be exempted from the Directive (Art.3). So from a regulatory point of view the resistance trait has been introduced with conventional breeding methods and consequently does not warrant any evaluation of its potential environmental effects. Moreover the traceability and labelling requirements do not apply to such products in the EU as they are linked to the GMO status. This example illustrates that rather the new traits introduced into crop plants and not the breeding techniques which lead to their introduction are responsible for the (adverse) consequences associated with a specific crop plant. At the moment this discrepancy in the regulatory system is unsolved and debates are under way to further address this issue.

Irrespective of the discussion of the regulatory issue in this study the focus is laid on the characteristics of an adequate risk assessment of NPBT crops. Therefore a short outline of general risk assessment requirements is given and further illustrated by the respective requirements laid down at EU level for GMOs for in EU Directive 2001/18/EC and Regulation (EC) Nr. 1829/2003, for plants with novel traits (PNT) in Canada and at international level in the Cartagena Protocol on Biosafety. Subsequently criteria are discussed which may be of help to address potential hazards associated with the application of NPBTs. Finally a number of cross cutting issues are discussed which are important for the evaluation of NPBTs.

3.1 Current Risk Assessment Requirements of GMOs

In the following table (Tab. 6) an overview is presented on the basic requirements which are particularly relevant for the first step of a RA, i.e. hazard identification. Just like for GMOs these requirements may equally be relevant for the risk assessment of products obtained with NPBT. Plants derived with NPBT may also serve different purposes (i.e. food use, feed use, industrial use, breeding purposes) and may be applied for different scopes of use (e.g. confined release, import & processing, cultivation). The RA of plants derived from NPBTs in general would have to cover potential human and animal health aspects as well as environmental effects, with case-specific adaptations of design governed by their specific scope of use.

*Table 6:
Overview on basic
requirements for
environmental and food
& feed risk assessment*

Basic RA requirement	Assessment issues (non exhaustive lists)
Molecular characterisation of the genomic modifications	● method used for transformation ● nature & source of vector used ● source of the genetic construct ● characterisation of differences at the DNA level between the modified organisms and the recipient organisms
Characterisation of the modified organism	● traits & characteristics modified ● sequences inserted/deleted ● expression of the insert

Basic RA requirement	Assessment issues (non exhaustive lists)
	● stability of the insert/trait ● (horizontal) gene transfer ● compositional changes ● phenotypic changes
Characterisation of health effects (food & feed safety)	● toxicology ● allergenicity ● nutritional effects
Characterisation of environmental effects	● persistence & invasiveness (incl. plant-to-plant gene flow) ● plant-to-microorganism gene transfer ● interactions with target organisms (e.g. development of resistance in target organism) ● interactions with non-target organisms ● impacts of the specific cultivation ● changes in management & harvesting techniques ● effects on biochemical processes and natural cycling processes of organic and anorganics materials

3.1.1 RA Requirements in the EU

In Europe a technique oriented notification system is established which means that the technique of modification which has been applied determines whether the obtained crop is subject to risk assessment according to the current GMO legislation or not (Ec 2001 Annex I). The authorisation system in the EU is case-specific implying that each GMO (i.e. each transformation event in a specific organism) has to be risk assessed separately.

The purpose of the biosafety regulations established for GMOs is to avoid adverse effect on human health and the environment (Ec 2001). Therefore a mandatory risk assessment needs to be conducted including an environmental risk assessment (ERA). This risk assessment needs to be based on an appropriate approach according to the general guiding principles as laid down e.g. in Annex II of EU Directive 2001/18/EC. The applicable principles are further detailed in the complementary Guidance Notes (Ec 2002). In short “the objective of an environmental risk assessment is, on a case by case basis to identify and evaluate potential adverse effects of the GMO, either direct or indirect, immediate or delayed, on human health and the environment”.

To conduct an ERA a procedure which is based on the following six steps should be carried out (Ec 2001): problem formulation including hazard identification, hazard characterisation, exposure characterisation, risk characterisation, evaluation of risk management strategies and overall risk evaluation. According to this approach and based on the following general principles an ERA should:

- Compare identified characteristics of the GMO and its use which have the potential to cause adverse effects to those presented by the non-modified organism.

- Be conducted in a scientific and transparent manner based on scientific and technical data.
- Be conducted on a case-by-case basis taking into account the type of GMO, its intended use and the potential receiving environment (incl. GMOs already in the environment).
- Analyse potential long-term effects (incl. accumulated effects of various consents).
- Identify need for risk management measures and the most appropriate measures to be used for mitigation
- Be reassessed in case new information on the GMO and its effect become available.

This general approach to ERA, as well as the its guiding principles are also the basis for the assessment of GMOs according to Regulation (EC) Nr. 1829/2003, which focuses on the protection of human life and health, animal health and welfare, environment and consumer interests (Ec 2003). The objectives pursued by EU Direction 2001/18/EC and by Regulation (EC) Nr. 1829/2003 are thus very similar. In addition to the protection of humans, animals and the environment in Switzerland the protection of biological diversity and its sustainable use are explicitly laid down in respective legislation (FrSV 2008). As some plants derived with NPBT may be associated with similar potential risks as GM plants the same protection goals would apply.

To provide guidance to the risk assessment conducted for notifications of GM crops in the EU EFSA has issued two separate guidance documents taking into account the different issues relevant for the risk assessment of the food and feed products and the environmental risk assessment. One of these guidance documents is addressing details of the environmental risk assessment of GM plants (EFSA 2010) and another guidance document is specifying the risk assessment of food and feed from GM plants (EFSA 2011). Some of the information requirements necessary for the procedure of an ERA are given in EU Directive 2001/18/EC and the EFSA guidance document is supplying details with respect to specific areas of risk to be addressed in the ERA (EFSA 2010). EU Regulation Nr. (EC) 503/2013 specifies requirements for the risk assessment for food & feed in a legally binding form (Ec 2013).

One of the common principles of the mentioned EFSA guidance documents is the comparative approach, which serves the purpose of identifying intended and unintended differences between the GM plants and its conventional counterpart taking into account natural variation (EFSA 2010 & 2011). For this purpose a case-specific comparative assessment of the compositional, phenotypic and agronomic as well as environmental characteristics is being conducted. Both food & feed RA as well as the ERA is based on information regarding the modified organism and its molecular characterisation. The respective information requirements are essentially the same for both types of assessment.

However the two guidance documents differ with respect to the specific areas of risk which ought to be paid special attention to in the risk assessment and the data which need to be produced for each specific area. For example in the GM food & feed risk assessment the allergenicity assessment plays an important role in addition to the assessment of potential toxic or anti-nutritional effects on

humans and animal consumers. On the other hand while in the environmental risk assessment e.g. the assessment of potential interactions with non-target organisms is a focal issue. However it should be noted that overlaps of both assessments exist, e.g. with the eco-toxicological evaluation, which is relevant for the assessment of potential effects on non-target organisms.

3.1.2 RA Requirements in Canada

Contrary to the EU Canada has adopted a product based evaluation system. In Canada a risk/safety assessment is required for plants with novel traits (PNT) and/or any novel livestock feed derived from plants with novel traits, irrespective of the method used to introduce the novel traits (CFIA, 2013). The concept of 'novelty' is not only used in the regulation of new varieties of plant species and new plant species introduced to the Canadian market (PNTs are regulated under Part V of the Seed Regulation) but also in the regulation of novel foods, novel aquatic organisms and new substances (CFIA 2014). Per definition a 'PNT is containing a trait not present in plants of the same species already existing as stable, cultivated populations in Canada, or is present at a level significantly outside the range of the trait in stable, cultivated populations of that plant species in Canada' (CFIA 2014). The determination of the novelty status of a new plant variety is decided upon on a case-by-case basis. Equally the novelty status of its derived food and feed products is evaluated separately and thus may be different.

A trait is considered to be novel when it has both of the following characteristics:

- it is "novel", i.e. not present in stable, cultivated populations of the plant species in Canada, and
- it has the potential to result in adverse environmental effects.

So far all genetically engineered plants have been considered to contain novel traits and have been assessed for environmental safety. Additionally the broad regulatory approach implemented with the novelty concept in Canada includes plants whose novel traits were introduced by conventional breeding or use of NPBT. However these crops constitute only a small minority of the regulated applications.

The objective of the Canadian regulation is to protect humans, animals and the environment. Similar to the two different foci of RA applied for GMOs in the EU (food & feed and environmental aspects) either an environmental assessment or a livestock feed assessment of PNTs is carried out. Again the two types of assessment are complementary to each other and some of the information used for the respective assessments is overlapping (e.g. description of novel traits and the modification).

For the environmental safety assessment of PNTs CFIA applies the following 5 criteria (CFIA 2008):

- potential of the PNT to become a weed of agriculture or be invasive of natural habitats
- potential for gene flow to sexually compatible plants whose hybrid offspring may become more weedy or more invasive
- potential for the PNT to become a plant pest

- potential impact of the PNT or its gene products on non-target species, including humans
- potential impact on biodiversity

Moreover the following issues have to be addressed by the applicant (CFIA 2008):

- the identity and the origin of the PNT
- the properties of the novel gene and gene products
- the relative phenotypic expression of the PNT compared to a similar counterpart, if respective differences are anticipated
- anticipated or known relative effects in the environment resulting from the release

Basically the applicant has to submit relevant information on the description of the PNT and its modification as well as on its biology and interactions in order for a safety assessment to be carried out.

3.1.3 RA Requirements in the Cartagena Protocol on Biosafety

The Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity (CBD) is an international agreement which aims “to ensure the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology that may have adverse effects on biological diversity, taking also into account risks to human health” (CBD 2000). By February 2014 166 countries have become parties to the CPB worldwide and thus are obliged to adhere to the standards laid down in the protocol. The basic principles and methodology for RA are laid down in Annex III of the protocol. In the past few years more detailed guidance on the RA of LMOs have been elaborated, including the so-called “road map for risk assessment” describing the general issues relevant for designing risk assessments.

With respect to RA the CPB focuses on:

- The protection of the biodiversity taking into account risk to human health.
- Risk assessments carried out in a scientifically and sound manner based on data of high scientific quality and relevance.
- Identification and consideration of uncertainty (*‘lack of scientific knowledge or consensus should not necessarily be interpreted as indicating a particular level or risk, an absence of risk, or an acceptable risk’* – Annex III.4) (precautionary approach).
- Case specific assessment of risks (case-by-case approach).
- Identifying changes between the LMO and its comparator (comparative approach).
- Reassessment in case of new information (iterative approach).
- Identification of risk management measures and strategies.

On a general level the approach to RAs according to the CPB is quite similar to the one implemented in the EU.

3.1.4 Relevance of the approach to GMO-RA for NPBT-crops

The CPB established an international framework for the RA and as such represents the minimal requirements to be fulfilled by all 166 parties of the CPB. Although elaborated later than many national regulations, the obligations according to the CPB introduced common principles for risk assessment to a majority of countries worldwide.

At first glance the product-based notification system in Canada seems to be different from the method-based system in the EU. Taking a closer look this difference turns out to be more linked to the question which organisms are subject to risk assessment rather than to the question on how a risk assessment is conducted. The information requirements and specific issues addressed in the course of the risk assessment are more or less the same in both systems. Differences in depth and focus of individual assessments are due to the specific GMO, its intended use and the respective receiving environment, but comparable risk assessment requirements apply.

It is evident that for risk assessments only general rules are established with regard to what data are necessary to be able to conduct a risk assessment 'in a scientifically sound and transparent manner' (CBD 2000). A key issue is the first step in ERA, i.e. the identification of potential hazards which might arise. Secondly data allowing for hazard and exposure characterisation in order to clarify whether the identified potential hazards actually do pose risks or not need to be provided. If risks are identified, it needs to be determined how they could be managed.

Of course this broadly formulated methodology leaves room for different interpretations and in practice has led to substantial differences and even controversies surrounding its application. Examples are for instance the discussion on whether the whole GMO should be the focus of the assessment or whether merely its new characteristic merit assessment, the application of the concept of substantial equivalence originally elaborated for food safety assessments in the ERA or the question whether an absence of evidence of adverse effects e.g. in laboratory tests constitutes evidence indicating safety and allows for dismissing tests at higher levels (for review see HILBECK 2011).

Such controversies can to a great extent be explained by the - often implicit - application of two different risk models: the causal assessment model and the risk model (c.f. ECNH 2012). In this respect the Ethics Committee on Non-Human Biotechnology (ECNH) in Switzerland has issued a statement clarifying that it 'unanimously supports the position that in case of GM plants we are not in a situation of total lack of knowledge, but of incomplete knowledge'. Thus the Committee recommends to base the risk assessment of GMOs on the so called 'risk model' which acknowledges that due to the complexity of situations and due to our limited human cognitive capacity only preliminary conclusions on the basis of available knowledge can be drawn (ECNH 2012). In this model the level of risk is calculated as the product of the probability of occurrence of the damage (also called exposure assessment) and the possible extent of damage (also called hazard characterisation). These two probabilistic aspects – the possibility of adverse effects happening and of the consequences of such effects – are generally the two basic components of risk as a concept (HILL 2005).

Many requirements in the European legislative framework for GMOs indicate that a risk model as described above is applied to the implemented ERA-approach:

- Acknowledgement of the irreversibility of effects resulting from the environmental release of GMOs (Ec 2001, recital 4)
- Consideration of the precautionary principle (Ec 2001, recital 8)
- Defined limited consent for the first 10 years (Ec 2001 Art.7.5. und Art 19.5)
- Concept of risk as a question of probability and the iterative character of risk assessment which requires reassessment in case of new information (Ec 2002, Ec 2001 Art.8 und Art.20)
- The analysis of cumulative long-term effects (Ec 2002, chapter 3.)
- The evaluation of potential direct, indirect, immediate and delayed effects GMOs may exert on human health and the environment (EU Dir. Annex II.A)
- Obligatory post market environmental monitoring (Ec 2001, recital 43) aiming at the confirmation of the assumptions of the ERA and the identification of effects not anticipated in the ERA (Ec 2001 Annex VII)
- Establishment of labelling & traceability requirements in order to facilitate firstly the withdrawal of GMOs from the market in case of unforeseen effects, secondly the monitoring of potential effects on the environment and thirdly the implementation of management measures (Ec 2003; recital 3)

Also the CPB contains elements which are clearly connected to the risk model: reference to the precautionary approach contained in the Rio Declaration on Environment and Development, the review of decisions, labelling requirements and monitoring (CBD 2000). In fact all three regulatory frameworks described above do contain elements which indicate that the risk model is applied as an underlying concept (e.g. monitoring requirements, consideration of uncertainties and review of decisions). The most stringent conditions in this respect however are laid down in the EU regulatory framework, which includes for instance traceability and labelling requirements, obligatory post market environmental monitoring including general surveillance (see above).

Most importantly the underlying risk model influences the practice of risk assessment. While the basic risk assessment requirements are essentially the same in the presented regulatory systems, the conduct of risk assessments and the conclusions drawn may vary between different countries.

Nevertheless the basic risk assessment requirements currently established for GMOs or PNTs can be considered adequate for the identification of potential risks of NPBT crops. Specific aspects relevant for the ERA of NPBT crops are discussed below (see chapters 3.2. & 3.3.). However the question whether NPBT crops have to be risk assessed in any case, depending on the techniques they were produced with or whether a case-specific approach is applied which bases this decision on the characteristics of the new trait ('Canadian model'), is a socio-political decision which has to be taken independent of the risk assessment procedure itself.

3.2 Criteria for the risk assessment of plants obtained with NPBT

It is evident that a comprehensive evaluation of the potential risks of a specific NPBT-crop cannot be based solely on generic considerations addressing only characteristics of the techniques or combination of techniques used to generate the respective NPBT-crop. Rather a case-specific approach is considered appropriate for the assessment of NPBT-crops (cf. VOGEL 2012).

Such an approach needs to be focused on the specific characteristics of the respective NPBT-crop and its interactions with human health and the environment exposed to the NPBT-crop. Furthermore the effects of products derived from such plants, e.g. food and feed prepared from such crops need to be considered. Additionally the consequences associated with the use of such crops, e.g. on the agricultural management, and any adverse effects resulting from such changes need to be addressed.

With a view to the different potential (crop) plants which are developed by NPBTs and their characteristics (see respective sections in chapter 2) this is comparable to the approach developed for a case specific risk assessment of GM-crops enshrined in the European regulation frameworks. For some relevant types of NPBT applications, among others for cisgenesis/intragenesis and site-directed mutagenesis applications resulting in the stable integration of new sequences, this was underpinned by recently published reviews (e.g. EFSA 2012a & 2012b, AGES 2011 & 2013).

As noted by previous reviews (see above references and VOGEL 2012) NPBT-crops are developed for different traits and application purposes. Additionally it can be expected that the exposure of human beings and the environment will differ significantly between different NPBT-crops and will involve different exposure pathways. Therefore the potential adverse effects associated with these NPBT-crops will be different as well.

However the necessary case-by-case assessment will among other considerations need to address aspects which are related to the specific plant breeding technique applied. Such aspects should also be considered to identify similarities and differences between a specific NPBT-crop on the one hand and comparable GM plants and crop plants generated with conventional breeding techniques on the other hand. Such a comparison will be valuable to identify specific assessment requirements for NPBT-crops. Additionally this analysis will help to identify areas of existing knowledge and experience which can support an assessment of NPBT-crops.

The following four criteria in this respect are considered to be specifically relevant for the risk assessment of plants obtained with NPBTs. Comparable considerations were also taken into account by EFSA for consideration of specific types of NPBTs (cisgenesis and intragenesis; ZFNs and other SSNs) (EFSA 2012a & 2012b). These aspects will be discussed in the subsequent chapter:

- Modifications introduced into the crop genome
- Knowledge and experience with the traits generated by application of NPBTs
- Presence of non-crop plant sequences
- Modification of gene expression

Furthermore a couple of other more general considerations apply for the assessment of NPBT-crops. These wider issues which are also important for the assessment of potential risks of NPBT-crops are introduced in Chapter 3.2.2 below.

3.2.1 Modifications introduced into the crop genome

Whether genomic modifications were introduced into the genome of a particular plant line in the course of application of NPBTs, and which type of such modifications were introduced is a crucial information for the design of appropriate risk assessment considerations. The reviewed NPBTs are characterised by a specific potential to introduce a diverse range of different genomic modifications. The following table 7 is providing a generalised summary of such modifications due to NPBT application. Specifics for different techniques are discussed below. However it needs to be taken into account that different NPBTs may be used in combination to address specific breeding goals. An overview on possible combinations is provided by Vogel (2012, Table 5 p. 77). An assessment of specific approaches therefore needs to consider the characteristics of all involved techniques.

Table 7: Overview on the potential of NPBTs to introduce intended and unintended genomic changes

NPBT	intended genomic modification by NPBT	unintended genomic modifications	recombinant DNA present in
Cell Fusion			
Protoplast fusion	fusion of parental genomes	rearrangements in parental genomes (chromosome numbers, structure)	-
Cytoplasm fusion	introduction of new plastid genomes	unintended genomic fusion	-
Marker Assisted Selection	-	natural variation	-
Tilling	selected random mutations	random mutations	-
Oligo-directed mutagenesis	targeted mutagenesis	off-target mutations	-
Nuclease-mediated site-directed mutagenesis			
SSN 1 & SSN2	targeted mutagenesis / deletions	off-target mutations	breeding intermediate
SSN3	transgenic insertions / deletions	off-target mutations	breeding product (heritable)
RdDM	- / (epigenetic modification)	off-target regulatory effects	breeding intermediate
Cisgenesis / Intragenesis	cisgenic / intragenic insertions	similar to transgenesis	breeding product (heritable)
Transgrafting	transgenic insertions in rootstock	similar to transgenesis (affected plant part)	part of breeding product / rootstock (non-heritable)
Techniques to support breeding			
Reverse breeding	-	similar to transgenesis / increased random variation	breeding intermediate
Seed production technology	transgenic insertions (maintainer line)	similar to transgenesis (maintainer line)	breeding intermediate (maintainer line)

NPBT	intended genomic modification by NPBT	unintended genomic modifications	recombinant DNA present in
Accelerated breeding	-	similar to transgenesis	breeding intermediate
Agroinfiltration s.s. / Agroinfection	-	transgenic insertions of episomal constructs	-
Transgenesis / floral dip	transgenic insertions	similar to transgenesis	breeding product (heritable)
Conventional Breeding	-	natural variation	-
Classical mutagenesis	random mutations (target)	random mutations (off-target)	-

Some genomic effects, i.e. integration of recombinant DNA constructs resulting in expression of specific traits, are similar to the ones introduced by GM technology (e.g. SSN3, floral dip, Cisgenesis/Intragenesis). These techniques generate additional genetic variation by introduction of additional genes/alleles into the parental genomes. However relevant differences to standard GM technology need to be considered, e.g. whether insertions are targeted to specific genomic locations (e.g. SSN3) or integrated randomly (floral dip, Cisgenesis/Intragenesis). Furthermore the genetic elements for these constructs may be derived from different organisms (parental crop species or other organisms) and recombined differently (e.g. Cisgenesis vs. other approaches).

The genomic effects for some other NPBTs of the techniques resemble the effects of traditional approaches of mutation breeding – however the mutations are mostly introduced at specific target sites in the NPBT-crops (ODM, SSN1/2) or selected in a targeted way from a random pool of mutations (Tilling). Protoplast fusion can lead to generation of breeding products with a radically changed genetic setup by fusion of naturally occurring, different genomes.

A group of techniques (e.g. Techniques to support breeding, MAS, agroinfiltration sensu strictu/agroinfection) is not supposed to introduce additional genomic variation into the available breeders gene pool (for definition see: Podevin et al. 2012), but is used to enhance other aspects of breeding processes (e.g. to reduce associated time/effort/cost requirements).

Some of the above approaches require that transgenic traits are introduced e.g. by GM technology to facilitate certain steps in the breeding process. VOGEL (2012) provided an overview on the different objectives to use GM technology in NPBT approaches (VOGEL 2012, Table 9, p. 84).

It is important to take into consideration if such modifications are present only transiently, in certain intermediate steps of the breeding process or present in a stable inheritable manner in the final breeding product (see table 8). The risk assessment in this respect needs also to take into account whether some (transgenic) modifications were introduced into the plant genome at a certain step of the NPBT procedure, but removed (fully or partly) subsequently – by segregation or otherwise. An assessment conducted for these NPBT-crops would need to confirm the absence of intermediary modifications in the final breeding product. This, however, is dependent on the ability to detect all different kinds of recombinant modifications which might be retained in the final breeding product (c.f. VOGEL 2012)

Table 8:
Types of NPBTs based
on presence of
recombinant DNA
sequences in NPBT
(modified from
PODEVIN et al. 2012):

NPBT-Types	NPBTs
Transient presence of transgenic DNA	SSNs1 & 2 ODM (synthetic oligos) Agroinfiltration s.s. (RdDM)
Stable introduction of recombinant DNA in breeding intermediates	SSNs 1 & 2 RdDM Reverse breeding Seed production technology Accelerated breeding
Stable integration of recombinant DNA	Cisgenesis Intragenesis Transcrafting SSN 3 floral dip
No recombinant sequences introduced	Cell Fusion Marker Assisted Selection Classical mutagenesis

An additional issue is whether such genomic modifications are present in all cells of the NPBT-crop or only in specific types of cells (e.g. somatic cells, reproductive cells) or plant parts (e.g. Transgrafting).

The outlined issues need to be addressed on the one hand for intended modifications, but on the other hand also concerning the potential of the NPBT to introduce unintended genomic modifications. With respect to the latter issue the state of knowledge on the nature of unintended effects which might be associated with a particular NPBT is important. Similarly important is information which supports the assessment of the level of uncertainty regarding possible unintended effects, which needs to be considered for a specific NPBT-crop.

Based on this information a comparison is possible whether similar genomic modifications would also occur during construction of GM plants and whether existing approaches developed for GMOs would also be appropriate for a risk assessment of certain NPBT-crops.

The information can also be used to consider whether genomic modifications of a certain type present in NPBT-crops, e.g. specific types of mutations, could also occur during conventional breeding approaches, e.g. as spontaneous or induced mutations or due to somaclonal variation.

However it should be noted that such a similarity cannot be considered indicative as regards the safety of a particular NPBT-crop without specific evidence. Evidence whether such mutations would occur at a different frequency during application of NPBTs compared to conventional breeding or whether any of these modifications would be genetically linked to desired traits can support the evaluation of NPBT-crops.

3.2.2 Presence of non-native sequences in NPBT crops

The issue whether specific NPBTs introduce non-native sequences into the context of a plant genome that does not contain similar genetic elements is regarded important for the risk assessment of NPBT-crops. Similar considerations are taken into account for the risk assessment of GMOs. EFSA (2012a & 2012b) regarded this issue also as crucial for the risk assessment of Cisgenic and Intragenic plants as well as for the assessment of SSN-applications, specifically SSN3 applications, which are designed to introduce additional genetic elements into the breeding product. The following table presents an overview on this issue, a discussion of this effect can be found in VOGEL (2012).

Table 9: Overview on the potential of non-native crop sequences to occur in NPBTs:

NPBT	Potential to introduce non-native crop sequences	Type of non native-sequences
Cell Fusion	+++	Genomic sequences originating from fusion partner genome
Marker Assisted Selection	-	-
Oligo-directed mutagenesis	++ (oligo-directed sequence changes)	Point mutations in target sequences (Base-substitutions), Off-target integration of synthetic oligonucleotides
Nuclease-mediated site-directed mutagenesis		
SSN 1 & SSN2	++ (insertion of random sequences)	Mutations in target sequences (Indels)
SSN3	+++ (intentional insertion of transgenes)	Transgenic constructs
RdDM	+ (transgenic inducer elements)	Unintentionally retained transgenic elements originating from primary modification
Cisgenesis / Intragenesis	++ (T-DNA sequences)	T-DNA border of cisgenic constructs/Intragenic constructs
Transgrafting	+++ (GM rootstock)	Transgenic constructs in modified plant part (e.g. rootstock)
Techniques to support breeding		
Reverse breeding	+ (transgenic inducer elements)	Unintentionally retained transgenic elements originating from primary modification
Seed production technology	+ (transgenes derived from maintainer line)	Unintentionally retained GM elements from maintainer line
Accelerated breeding	+ (transgenic inducer)	Unintentionally retained transgenic elements originating from primary modification
Agroinfiltration s.s. / Agroinfection	+ (rare integration events)	Unintentionally integrated recombinant T-DNA elements
Transgenesis / floral dip	+++ (transgenic constructs)	Intentionally inserted transgenic constructs
Conventional Breeding	-	-
Classical mutagenesis	+++ (all types of random mutations)	Random mutations

In this respect the knowledge needs to be evaluated which is available on the source organisms of such sequences, on the function(s) of such sequences, on the history of use in crop plants or in products similar to NPBT-plant products and on experience available regarding safe use or adverse effects associated with organisms containing similar genetic elements.

Information concerning non-native sequences introduced into NPBT-crops can be used to identify similarities of such NPBT-crops with GM plants. Such similarities would indicate similar risk assessment requirements. Comparability with conventionally bred crops in this respect however is limited. Occurrence of such sequences, specifically sequences introduced as effect of transformation methods, is not a relevant issue for conventional breeding methods (EFSA 2012a). However conventional breeding based on intentionally mutagenised crop varieties may exhibit a broad range of genetic alterations that would not likely occur in populations untreated crop plants.

3.2.3 Knowledge and experience with the traits developed by application of NPBTs

The non-exhaustive review provided in Chapter 2 clearly illustrates that a very broad range of different crop traits is being developed by breeding approaches based on NPBT methods. As indicated above the different crop/trait combinations will be associated with a specific potential to exert (adverse) effects on human health and the environment as determined by the phenotype of the specific NPBT-crop and the exposure of the environment to it. Risk assessment therefore has to be designed in a case-specific manner (cf. among others EFSA 2012a & 2012b, VOGEL 2012). A case-specific approach is also taken by the regulation system in place in Canada (see Chapter 3.1.2) for “plants with novel traits” that are determined to be novel as well as associated with a potential for adverse effects.

In the framework of a case-specific approach the availability of sufficient knowledge on the modified genes and their function as well as experience with the traits generated by NPBTs is an extremely relevant issue for the assessment of NPBT-crops. This is also recognized by EFSA’s concept of “history of safe use” which is regarded as an important element in the comparative risk assessment for GM plants (EFSA 2011) as well as applications of specific NPBTs (cf. EFSA 2012a & 2012b). This criterium builds also on the concept introduced by OECD (1993) for a comparative approach to the environmental risk assessment of GM plants based on appropriate comparator plants with well described biology: Previous knowledge and experience with the crop plant, the environment, the trait and the interactions and familiarity with any of these elements is regarded important to facilitate risk/safety assessments (OECD 1993). However familiarity with any of these aspects does not determine whether a new combination is either safe or risky. In particular with regard to this aspect there have been misinterpretations regarding the application of this concept.

It is also important to verify whether the available experience and data e.g. as drawn from conventionally bred crops are appropriate to address the specific risk relevant issues encountered with applications of NPBTs. A critical appraisal of the knowledge on the origin and function of genes affected by NPBTs and the resultant traits needs therefore to be conducted. As outlined above similar requirements apply for the assessment of modifications to (endogenous) genes or other genomic elements by NPBTs.

Sufficient knowledge about the source(s) of the newly introduced genes or modifications (i.e. source of trait) is therefore an important piece of information for the risk assessment, in particular with respect to the food safety evaluation. EFSA distinguishes the following aspects:

- The comparator/donor plant has a history of cultivation and consumption by humans (e.g. variety, landrace, wild relative).
- The comparator/donor plant has no history of consumption by humans, but has been used in conventional plant breeding.
- The comparator/donor plant has not yet been exploited for variety development, but there is knowledge of the gene family in terms of the structure and function of the protein they encode.
- The comparator/donor plant has not been exploited yet for variety development and gene family and the mode of function of the protein is not well established.

For this comparison it is important to consider whether the gene or its regulatory sequences have artificially being altered in a specific way or whether the expected exposure to the NPBT-crop can be regarded comparable (cf. Efsa 2012a).

3.2.4 Modification of gene expression

Modification of crops by NPBTs can result in various changes in gene expression in the NPBT and consequently in the phenotypic characteristics of the NPBT. These changes can be relevant as regards potential adverse effects and should be considered in the overall design of a risk assessment approach. For a significant number of NPBT approaches information regarding the modification of gene expression is a very important aspect (cf. EFSA 2012a).

However note should be taken that different effects need to be considered in this respect:

- Some applications aim intentionally to modify the expression of a specific endogenous target gene or facilitate the expression of a modified target gene product usually not present in the genome of the respective crop species. Examples for these applications are NPBTs which directly result in expression of additional genes (e.g. certain approaches of SSN3, Cisgenesis/Intragenesis, transgrafting, agroinfiltration, floral dip). Also changes in expression patterns of target genes facilitated by introducing additional copies of the target gene or changes to the regulatory elements of target genes need to be considered, as well as introduction of (mutated) gene alleles leading to expression of new traits. Such modifications may be generated or introduced by e.g. MAS, ODM, SSNs, TSBs, etc.
- Intentional changes in single target genes may also be introduced, by silencing or activating the endogenous expression of these target genes by different approaches. These include the introduction of regulators of gene expression (regulatory proteins or RNAs) by application of NPBTs among them SSN3, Cisgenesis/Intragenesis, transgrafting, agroinfiltration, floral dip, RdDM.

- Other intended effects target the modification of general regulation pathways for gene expression, with broader effects on global gene expression in specific cell types. This can be achieved by introduction of alleles for specific global regulatory proteins or expression of RNAi's which influence key elements of global regulation pathways (e.g. by SSN3, Cisgenesis/Intragenesis, transgrafting, agroinfiltration, floral dip, RdDM). Modification of global epigenetic regulation of gene expression in a NPBT-crop may be associated with a potential for unintended adverse effects (HEINEMANN et al. 2013). Such a potential needs to be scrutinised appropriately.
- On the other hand it needs to be considered whether a specific NPBT approach is associated with indirect effects on gene expression, e.g. (unintended) impacts on the expression of the modified trait or effects on expression of independent traits (cf. HEINEMANN et al. 2013).

Effects on gene expression are commonly associated with phenotypic changes, i.e. they may result in compositional or developmental changes, which can be associated with adverse effects on health and/or environment. Also effects concerning the way such crops will respond and adapt to environmental stress may be caused by effects of modifications by NPBTs on gene regulation.

As noted by EFSA (2012a) conventional breeding is also expected to result in changes in genome-wide gene expression patterns. However this is not indicative that changes in gene expression due to modifications by NPBTs can be considered negligible with regard to potential risks. Information concerning changes in gene expression in NPBT-crops therefore should to be assessed with a focus on biologically relevant parameters or patterns indicative of adverse effects. Likewise transgenic plants are assessed for compositional and phenotypic parameters which are influenced by changes in cellular gene expression levels (c.f. EFSA 2010 & 2011).

3.3 Wider issues concerning risk assessment of NPBT-crops

3.3.1 Comparators for risk assessment

The current approach for risk assessment for GM-crops and for PNT-crops in Canada is based on the comparative approach. This involves comparison of the characteristics of a modified crop with a non-modified crop used for comparable applications, e.g. in agriculture. To be able to implement a comparative approach two types of considerations need to be taken:

- Definition of an appropriate “unmodified” comparator.
This is usually a crop line which is closely related to the assessed crop (e.g. a GM-crop or a NPBT-crop). For GM-crops a so-called conventional comparator is recommended – typically a crop line with a similar genetic background (e.g. an isogenic line) which was not genetically modified (EFSA 2011). The choice of an appropriate comparator line is decisive for the ability to adequately identify and assess the specific characteristics of a modified crop.

- Choice of a scenario for conducting the comparative approach.

This consideration is necessary to define a framework of the comparison. For agricultural applications this is based on choice of the type of agricultural which is taken into comparison with an intended application of NPBT crops.

As regards the first question of appropriate comparator plants, certain difficulties with some techniques are obvious.

For certain NPBT-applications “conventional” counterparts are difficult to define. E.g. a conventional counterpart has not been defined for trans-grafting since the grafting technique has not yet been evaluated regarding the current EU standards of the comparative approach in risk assessment (AGES 2013). With other applications, e.g. SPT, it is not clear whether only the non-GM breeding product and not any modified breeding intermediates need to be considered concerning the choice of a comparator.

In-depth discussion of such aspects has only started and no conclusive results are available at present. However the difficulties some of the aspects associated with need to be further addressed since the availability of appropriate comparators is a crucial requirement for a meaningful risk assessment of NPBT-crops.

Also the choice of appropriate agricultural application scenarios is a complex issue with manifold effects for the conduct and interpretation of risk assessments for NPBT-crops.

Different types of existing agricultural management systems are prioritising different objectives (e.g. maximising production and profitability vs. minimising external inputs and reducing environmental effects vs. societal objectives e.g. protection of agricultural traditions and structures, etc.). Such systems are characterised by their different management practices. Common examples of such scenarios are e.g. input-based production systems which maximise production yields, extensive conventional production which tries to reduce external inputs (fertilizer and pesticide use), small scale production of high value products (e.g. seed material, organic products).

The effects of these different production systems on existing protection goals concerning human health, the environment and relevant societal issues can be quite distinct. In turn different baselines will need to be considered for the comparison with effects of (potential) application of NPBT-crops.

The above question however is not new. A similar discussion is ongoing for GM technology for quite some time (e.g. SCHULTE & KÄPPELI 2000). Aspects from this discussion will also be relevant and informative for the considerations taken for NPBT-crops.

Some aspects of this question need to be addressed at a technical level, e.g. as regards identification of adequate assessment endpoints, generation of appropriate data for comparison, etc. Others however will have to be decided at a political level, e.g. as regards triggers for regulation, decisions on which protection goals will apply for specific technologies, etc.

3.3.2 Assessment of Uncertainties Associated with NPBTs

The definition of risk according to Directive 2001/18/EC and the Guidance Notes supplementing Annex II to Directive 2001/18/EC for GMOs indicates the relevance of assessing the magnitude of the consequences of hazards and the

likelihood of adverse effects. Both aspects are associated with uncertainties (EFSA 2010), which need to be addressed in the framework of the environmental risk assessment. However such uncertainties are often due to insufficient knowledge and particularly an absence of data essential for the risk assessment. This is similarly relevant for the assessment of NPBT-crops.

For newly developed techniques an initial uncertainty is whether comparable risk assessment approaches would be sufficient to comprehensively address NPBT techniques. Evaluation of NPBTs by different institutions and national and international bodies seems to indicate that the range of issues which will need to be addressed for NPBTs is comparable to the issues encountered by GM-crops (among others OECD 2014, LUSSE & DAVIES 2013, EFSA 2012a & 2012b, PODEVIN et al. 2012, VOGEL 2012).

However a lack of knowledge on the mode of action of certain types of modifications, the modified traits and specifically on unintended effects are also relevant issues for the case-specific evaluation of NPBT-crops.

This is partially due to the early stage of development of some NPBT-approaches (see LUSSE et al. 2012, VOGEL 2012). While some applications, e.g. herbicide tolerant crop varieties developed by ODM, are already marketed in some countries and some applications are near to commercialisation, other are still at a stage of scientific research and proof of concept.

A recent review by VOGEL (2012) is confirming that little data is available regarding the safety of phenotypic characteristics and specifically the environmental effects of NPBT-crops relevant for risk assessment.

However against the background of further rapid development of NPBT-crops (e.g. PARISI 2013, LUSSE et al. 2012, VOGEL 2012) and the general interest of regulators in these techniques the issue of (environmental) risk assessment of NPBT-crops is attracting increased interest (OECD 2014). As a consequence the data base available from research and development and applied science including biosafety research will expand. The available information however will need to be evaluated for its relevance and significance as regards risk assessment requirements. This is noticeable in an increase in the number of different reviews analysing biosafety aspects related to NPBTs (see table 10 below).

*Table 10: Examples of publications addressing biosafety-aspects of NPBTs
List is not exhaustive and focuses on publications providing a synthesis of relevant scientific evidence.*

NPBT	Publications cited in Vogel (2012)	Additionally published papers/reports
Oligo-directed mutagenesis	LUSSE et al. 2011, BREYER et al. 2009, SCHAART & VISSER 2009, COGEM 2006a,	COGEM 2010, LUSSE & DAVIES 2013, PODEVIN et al. 2012
Nuclease-mediated site-directed mutagenesis		
SSN1 & SSN2	LUSSE et al. 2011, COGEM 2009a	PODEVIN et al., 2013; PUCHTA & FAUSER, 2013, PAUWELS et al. 2013, LUSSE & DAVIES 2013, PODEVIN et al. 2012
SSN3	EFSA 2012b, Lusser et al. 2011, COGEM 2009a	PODEVIN et al., 2013; PUCHTA & FAUSER, 2013, PAUWELS et al. 2013, LUSSE & DAVIES 2013, PODEVIN et al. 2012, WEINTHAL

NPBT	Publications cited in Vogel (2012)	Additionally published papers/reports
		2010
RdDM	LUSSEr et al. 2011, COGEM 2006a	AGES 2013; RAMESH 2013, PODEVIN et al. 2012, HEINEMANN et al. 2013, AUER & FREDERICK 2009, LUNDGREN & DUAN 2013
Cisgenesis/ Intragenesis	EFSA 2012a, LUSSEr et al. 2011, PRINS & KOK 2010, JACOBSEN & SCHOUTEN 2009/2008/2007, SCHAART & VISSER 2009, Kok et al. 2008, Russel & Sparrow 2008, COGEM 2006b, De Cock Buning et al. 2006, Giddings 2006, Myskja 2006, Schouten et al. 2006a/b/c, Schubert & Williams 2006, Krens 2005, Rommens 2007, Rommens et al. 2007	PODEVIN et al. 2012; LUSSEr & DAVIES 2013, AGES 2012
Transgrafting	Hemmer et al. 2009, SCHAART & VISSER 2009, YOUK et al. 2009, COGEM 2006a, Yi et al. 2006, VIGNE et al. 2004;	Lemgo et al. 2013, AGES 2013, LUSSEr & DAVIES 2013, PODEVIN et al. 2012, Smolka et al. 2010, Nagel 2010, HAROLDSEN et al. 2012a & 2012b
Techniques to support breeding		
Reverse breeding	LUSSEr et al. 2011, SCHAART & VISSER 2009, COGEM 2006a	AGES 2013, LUSSEr & DAVIES 2013
Seed production technology	USDA 2011	
Accelerated breeding	SCHAART & VISSER 2009	LUSSEr & DAVIES 2013
Agroinfiltration s.s./ Agroinfection	LUSSEr et al. 2011, SCHAART & VISSER 2009, COGEM 2006a	AGES 2012, LUSSEr & DAVIES 2013
Transgenesis/floral dip	-	AGES 2012, LUSSEr & DAVIES 2013

For some NPBT-applications the uncertainties associated with potential risk issues are far from being resolved yet (cf. individual sections in chapter 2 of this report, AGES 2012, 2014, VOGEL 2012). This is due to the quite limited availability of relevant scientific data to address such aspects. The current situation of insufficient knowledge associated with certain areas should be appropriately taken into account, e.g. by applying requirements to identify and assess such uncertainties similar as for GM plants (EFSA 2010).

To support the comparisons being made with either GM crops or conventionally bred crops, relevant data for these applications needs to be taken into account or established if necessary. For some issues (unintended effects, effects of insertional mutagenesis) current efforts are underway to address these issues in a general way, including comparison of NPBTs with conventional breeding approaches and GM technology (CERA, CFIA pers. communication)

The attention to potential risk issues can also be helpful to identify crucial issues for the assessment of particular techniques or combination of techniques (EFSA 2012a & 2012b, AGES 2012 & 2013, VOGEL 2012). This way certain areas could be identified which need further attention, among them:

- The potential for unintended effects associated with different NPBTs.
- Effects of the potential instability of traits developed e.g. by epigenetic engineering using RdDM-approaches.
- Interactions between GM rootstock and non-modified scion in transgrafting, particularly the transmission of molecules (e.g. proteins, RNA) from the GM rootstock to the scion and their influence on gene expression. Relevant factors (e.g. efficiency of transport, distance, accumulation, size, charge) are associated with considerable uncertainties, however these issues are deemed to be highly relevant for assessment (LUSSEK et al. 2011).

3.3.3 Potential of long-term and indirect risks due to agricultural use of NPBT-crops

An element of ERA as applied for GM plants according to Directive 2001/18/EC is an analysis of the "cumulative long-term effects relevant to the release and the placing on the market". With a view to the traits targeted by NPBT approaches to plant breeding it is apparent that some are target to similar objectives than respective development of GM plants, e.g. induction of tolerance to broadband herbicides, resistance to specific pathogens, compositional changes, etc. Some of these risks are associated with the changes in agricultural management when a NPBT-crop is cultivated, rather than on the direct effects of the modification. Indirect and delayed effects may also result from unintended effects of modifications by NPBTs or result from stability issues of the modifications and traits in NPBT crops.

Large scale agronomic application of crops with traits like herbicide tolerance or pathogen resistance may have indirect ecological impacts that need to be assessed specific information sources and techniques. Data generated according to other regulation requirements, including e.g. variety registration are not fully appropriate for this purpose. Rather methodologies as recommended for the assessment and monitoring of relevant baselines and subsequent changes for GM plants (EFSA 2010) should be considered for long-term effects and indirect effects of NPBT-crops with traits that may lead to such effects.

4 SUMMARY AND CONCLUSIONS

Features of New plant breeding techniques (NPBTs) considered in this study

Several open questions concerning the application of NPBTs to facilitating crop breeding are currently discussed, primarily the issue whether these NPBTs are subject to present regulation frameworks, e.g. as implemented for GM crops. A different line of discussion is focusing on questions related to the biosafety of NPBT-crops. This study analysed biosafety aspects for a number of different NPBTs, thus addressing an aspect that received considerably less attention in past years than issues concerning the regulatory status of NPBT-crops.

The following conclusions can be drawn from the analysis presented in this report:

- The individual NPBTs are indeed very different in approach and characteristics. According to these characteristics some NPBTs are applied to develop a wide range of different products.
- Some NPBTs are used to modify the genomic DNA of the targeted crop species in a specific way in a stable and heritable form. Other NPBTs are aimed to change gene expression in the target plants – by transient expression of non-integrated genetic elements or modification of epigenetic regulation of gene expression (e.g. agroinfiltration/agroinfection, RdDM). A third group of NPBT-applications (e.g. MAS, TSBs like reverse breeding, accelerated breeding, seed production technology) are applied as tools to facilitate selection or other breeding processes. Genetic modification of a breeding intermediate may be necessary to achieve the breeding objective, e.g. with TSBs – however the final breeding product is not meant to contain these GM modifications.
- A striking feature is that NPBTs are mostly used in combination. To develop products single NPBTs thus are combined with other NPBTs, with GM technology and conventional breeding approaches.
- As regards the products developed by NPBTs some are very specific and cannot easily be generated by other approaches (e.g. targeted mutation and targeted integration of recombinant constructs, reverse breeding of parental lines for reconstituting elite hybrids and rapid accelerated breeding in crops like fruit trees).
- Other products – notably NPBT-crops with traits that render them resistant to certain broadband herbicides – are less specifically linked to NPBTs. Similar crop lines were developed by means of GM techniques and also by conventional breeding in a few crops species. As their phenotype and use is comparable, a similar potential for risks is characteristic for these crops - which should be addressed by a comparable risk assessment approach.
- For each NPBT the report compiles risk issues corresponding to the characteristics of the type of modifications. The different potential risks can be associated on the one hand with the intended modifications (e.g. introduction of alleles/mutations/regulatory effectors leading to traits which may be also connected to adverse effects). On the other hand potential adverse effects can result from unintended effects resulting of application of NPBT. Some of these unintended effects are due to methods like in vitro cell/tissue cultivation which need to be used in the process of NPBT, but which are not specific to a certain NPBT. Similar effects may also occur upon application of these techniques in conventional breeding.

- GM techniques are used directly (Cisgenesis/Intragenesis, transcrafting, floral dip) or indirectly (agroinfiltration, TSBs, Nuclease-mediated site-directed mutagenesis by SSNs), as tools in the course of NPBT processes.
- With indirect applications of GM technology the introduced modifications are intended to be present only in breeding intermediates, and not in the final breeding product. The final product then should be devoid of modifications specific for such a NPBT. This is limiting the possibilities for specific detection of a certain NPBT-crop, an issue which is also relevant for risk assessment and monitoring.

Comparison of NPBTs with GM-technology and conventional breeding

When compared with either conventional breeding based on random mutagenesis or GM technology some NPBTs show analogous features of the latter approaches. However, it needs to be noted that these techniques are not strictly “similar” to one another.

The specifics of NPBTs (e.g. different targeting of insertions or mutation, different frequencies of off-target effects at certain genomic locations, different possibilities to introduce/select certain traits) should be considered in a risk assessment with a view to their specific consequences. A careful assessment of these specifics is a prerequisite for an appropriate design of the risk assessment approach. It can also be used to base the assessment of specific issues of NPBT-crops on existing experience. On this basis appropriate elements from the approaches which are used for conventionally bred crops or GM crops should be selected for NPBT-crops (assessment based on known familiarity, assessment according to incomplete knowledge).

General framework for risk assessment of NPBT-crops

Thus far biosafety considerations conducted for NPBT crops have indicated that the general approach developed for the risk assessment of GM crops in principle would also be appropriate to address the currently identified risk issues for NPBT-crops. Also the basic principles implemented in relevant biosafety regulation frameworks – European legislation, Cartagena Protocol, Canadian “Plants with Novel Traits” regulation - are considered to be appropriate for NPBT-crops, taking into account that for some NPBT applications only insufficient knowledge is available as regards their potential for adverse effects.

Specifically the principles of case-specific risk assessment, requirement of a scientifically based risk assessment according to the risk model and application of the precautionary principle would also be appropriate for NPBT-crops.

Specific considerations for risk assessment of NPBT-crops

The case-specific risk assessments should take into account the specific characteristics of the respective NPBT-crop. The report at hands suggests that four aspects should be specifically considered. Some of these aspects are tightly connected to features of the different NPBT-methods used. For other aspects the connection with the used NPBT-methods is less evident, e.g. for traits that may not be exclusively developed by application of NPBTs:

- the modifications of the genome due to NPBTs,
- the potential to introduce into the NPBT-genome (recombinant) DNA which is ordinarily not found in this crop species,

- the traits generated by NPBTs, and
- modifications to gene expression due to the NPBT or the trait(s) introduced by use of NPBTs.

As regards the different modifications of the genome introduced by NPBTs the report notes that a general discussion of their safety would not be appropriate. The discussed NPBT-approaches are aimed to achieve a range of different types of modifications, some characterised by a specific level of targeting, e.g. to introduce certain kinds of mutations. NPBT-approaches are also associated with specific potential to induce unintended effects. A characteristic feature of a number of NPBT-applications is that transgenic constructs are only present transiently (e.g. with agroinfiltration), in parts of the breeding product (e.g. transgrafting) or only during intermediary breeding steps. The assessment thus needs to take in account that final breeding products and intermediary breeding lines need to be considered differently.

Safety considerations should be based on the available evidence for a specific type of modification for the NPBT-approach in question taking into account experience with comparable other breeding methods and the particular differences between the compared situations.

Different NPBT-approaches are also characterised by a specific potential to introduce certain non-native sequences into the genomes of the resulting NPBT-crops. Again this potential and its significance need to be evaluated on a case-by case basis in comparison with GM and conventional breeding approaches.

NPBT-applications aim to develop crops with diverse traits – which might or might not be comparable to traits which can be developed with other breeding techniques. Specific assessment requirements should therefore be based on a critical appraisal of the available knowledge on the trait in question and the familiarity with the effects of such traits when used in agriculture and food production. For some traits due to modifications by NPBTs the availability of relevant evidence and thus the level of familiarity which can be deduced from existing experience may be limited. In such situations an appropriate risk assessment would be needed.

As regards modification of gene expression the report notes that the objective of a range of NPBT applications is to directly influence the expression of target traits. This is done via methods, e.g. RNAi-mediated silencing of gene expression, which may be associated to unintended effects.

Wider issues concerning the risk assessment of NPBT-crops

As issues which are crucial for the assessment of NPBT-crops the report specifically highlights the importance which protection goals are considered to design a risk assessment framework for NPBT-crops. Choice of protection goals will influence the definition what is considered a potential adverse effect associated with NPBT-crops (as well as other breeding products) and provide guidance for setting the scope of hazard identification as a first step in risk assessment.

Another difficult issue is to determine which NPBT-crops need to be subject to risk assessment requirement and what are the triggers for this decision. This is not solely a technical question open to scientific answers, but is also influenced by particularities of the existing regulation frameworks and political decision making. With regard to such questions also the Canadian model of product-based regulation is not free from difficulties and ambiguities.

Another important issue is the apparent lack of knowledge with several NPBT approaches. However the availability of appropriate (case-specific) scientific data is crucial for an adequate risk assessment of NPBT-crops with a potential for adverse effects or an unknown level of risk for unintended effects.

The general challenge is to keep up with the rapid pace of development of NPBT-crops, address the relevant risk issues by appropriate (biosafety) research and provide a synthesis of the existing information relevant for biosafety as an input for risk assessment. Information available from other regulation frameworks like variety registration will not be fully appropriate for risk assessment requirements. This is exemplified by the European experience with GM crops and the Canadian PNT-system.

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ANNEX 1: CLASSIFICATION SCHEMES FOR NPBTs

Several recent reviews attempted to provide a categorisation of the NPBTs, starting from the range of NPBTs which was discussed by the (N_TWG 2011) without a further hierarchical order.

One of these classification scheme was reviewed by a recent study addressing the regulatory challenges presented by NPBTs (PODEVIN et al. 2012). This study addressed the questions, whether the existing frameworks are fit for the purpose of regulating new techniques and whether the current regulatory approach implemented for GM crops would be proportional for to the risks associated with NPBTs. However PODEVIN and colleagues (2012) did not entirely focus on the issue, whether current regulations are appropriate to prevent harm caused by the application of NPBTs, but also discussed whether the existing frameworks would stimulate the development of innovative products based on new biotechnological plant breeding techniques and could build consumer trust into such products.

Focusing on the first of these issues PODEVIN et al. (2012) developed a different categorization scheme, which is dependent on the level of integration of recombinant DNA into the genome of a NPBT-crop (Tab. A1.1). The classification put forward is particularly focused on the issue whether individual NPBTs employ in vitro recombined nucleic acids and DNA delivery methods which are common in GM technology. A further criterium of consideration is whether NPBT-crops retain recombinant DNA-inserts transiently, or in intermediate steps or trans-generationally (i.e. inherited stably during further reproduction).

Tab. A1.1: Groups of NPBTs according to PODEVIN et al. (2012):

Group	NPBT-Category	Respective NPBTs involved
1	Transient introduction of recombinant DNA	● ZFN types 1 & 2 ¹ ● ODM ● Agro-infiltration (sensu stricto)
2	Transient introduction of recombinant DNA as an intermediate step in development	● ZFN types 1 & 2 ¹ ● RdDM ● Reverse breeding ● Accelerated breeding (early flowering)
3	Stable integration of recombinant DNA	● Cisgenesis ● Intragenesis ● Transcrafting ● ZFN type 3 ²

¹ ZFN1: site specific mutations by non-homologous random repair; ZFN2: induction of desired point mutations by homologous repair mechanisms.

² ZFN3: targeted delivery of transgenic insertions by homologous recombination.

Whether recombinant DNA is present transiently, in breeding intermediates or stably in NPBT-crops and their products is crucial for the categorisation according to PODEVIN et al. (2012) above. However this consideration is also highly relevant for a risk assessment conducted for a specific NPBT-crop. Therefore

the classification according to PODEVIN et al. (2012) is specifically highlighting this relevant dimension of a risk assessment of NPBTs.

Another type of classification was used e.g. by LUSSEr et al. (2012) and SCHAArt et al. (2009). This scheme is based on a broad set of criteria, involving the following considerations (for details see LUSSEr et al. 2012):

- Rationale for application of a specific NPBT
- Methodology of NPBT (types of molecules used for introduction of modification, method of modification, target tissues of modification process)
- Process of modification (molecular processes triggered, intermediate products involved in NPBT scheme)
- Characteristics of NPBT product (nature of change in genome, relationship to conventional breeding/natural mechanisms, associated off-target effects, possibility of detection)

Based on such criteria the following groups were identified (see Tab. A1.2) as a means for a more structured approach to discussion and to simplify evaluation of NPBTs. The discussion outlined in LUSSEr et al. (2012) however did not focus on the risk assessment of NPBTs but rather on the question, whether such NPBTs are subject to the regulation frameworks for biotechnology derived crops which exist in different countries (e.g. Argentina, Australia, Canada, EU-Member States, Japan, South Africa and USA).

Tab. A1.2: Groups of NPBTs according to LUSSEr et al. (2012):

Group	NPBT-Class ¹	Respective NPBTs involved
1	Site specific mutagenesis ²	● ZFN ● TALEN ● Meganucleases ● ODM
2	Cisgenesis & Intragenesis	● Cisgenesis ● Intragenesis
3	Breeding with transgenic inducer line	● RdDM ● Reverse breeding ● Accelerated breeding (early flowering)
4	Grafting techniques	● Transcrafting
5	Agro-Infiltration techniques	● Agro-infiltration (sensu stricto) ● Agro-infection ● Floral dip

¹ MAS and Protoplast fusion were not discussed specifically in LUSSEr et al. (2012) and would not belong to the outlined classes.

² CRISPR-Cas-Nucleases were not discussed as site specific mutagenesis techniques in LUSSEr et al. (2012, but would belong to Group 1 according to their specifics.

The considerations presented by LUSSEr et al. (2012) add other relevant dimensions required for a comprehensive risk assessment of NPBT crops.

ANNEX 2: OVERVIEW ON THE RISK ASSESSMENT CONSIDERATIONS FOR NPBT-CROPS

The following table A2.1 is presenting an overview on issues relevant for the problem formulation for the respective risk assessment of NPBT crops. The table is complementing the core considerations presented in Table 2 to address specific characteristics of NPBTs. Additions are twofold: First of all additional detail is added to the issues already introduced in Table 2 regarding possible outcomes of the consideration listed in the second column. Additionally considerations addressing characteristics of the crop plant species which is used for breeding and the characteristics of the receiving environment are added. These considerations are not specifically related to the NPBT-technology involved in development of a crop. However these aspects are highly relevant for the outcome of the risk assessment for a specific novel crop and thus also very important for the assessment of NPBT-crops.

Tab. A2.1: Comprehensive overview on the risk assessment considerations applicable to NPBT crops:

Categories for consideration	Issues for Consideration	Outcome
Intended modification by NPBT	1) Is a genetic modification introduced intentionally into the breeding product?	No/Yes (address 1.1 & 1.2)
	1.1) What kind of genetic modification is introduced?	Which gene(s)/regulatory element(s) are targeted?
	- Targeted mutation in a genetic element - Non-targeted mutation(s) in plant genome - Knock-out of native gene(s) - Introduction of modified gene(s) - gene "knock-In"	- Intended range of random mutations (single/many) - Which genes are disabled (1/many)? - Which genes/transgenes are introduced (1/many)?
	1.2) How stable are the introduced genetic modifications?	Transiently present/Stably present in breeding intermediate / Stably inherited in crop
		No/Yes (address 2.1 & 2.2)
	2) Are epigenetic modifications intentionally introduced in the breeding product?	
	2.1) What kind of epigenetic modification is introduced?	Methylation/Histone Acetylation/RNAi-Induction
	- Which epigenetic mechanism is targeted? - What is expected effect of epigenetic regulation? - Duration of the intended epigenetic regulation?	- Specific regulation/Silencing of gene expression, post-transcriptional regulation/Cell-wide regulation (e.g. developmental program, etc.) - Transient/Transgenerational

	What is the target cell type for epigenetic effect?	● Somatic tissue/Reproductive tissues
	2.2) Is a genetic modification necessary to establish the epigenetic effect?	● Transiently present/Stably present in breeding intermediate/Stably inherited in crop
Potential unintended effects of the used NPBT	1) Are genomic changes introduced at the modification site?	● No / Yes
	2) Are off-target modifications induced?	● Mutation(s) / Transposon mobilization
	3) Are (epigenetic) effects on gene regulation induced?	● Transiently / Transgenerational
	4) Are non-plant sequences introduced into the breeding product?	● No / Yes
	5) Which kinds of uncertainties may be associated with the breeding techniques used?	● Type and extent of uncertainties
	● Unintended phenotypical effects associated with NPBT?	
	● Movement of novel molecules between plant parts ● Adventitious reproductive functions established	● e.g. metabolites, RNA, proteins ● e.g. suckers, shoots grown from GM rootstock, which may produce flowers
Characteristics of the targeted traits	1) Source of trait	● Familiar /Non-familiar
	2) Function of trait(s)	● e.g. Insect resistance / Herbicide tolerance (HT) / pathogen resistance / compositional change / stress resistance
	3) Mode of action of trait	● Regulatory function / Native function / Non-native function
	4) Type of trait	● Loss of native function / Introduction of new function(s)
	5) Trait stability	● Transiently present / Stably inherited
Characteristics of the targeted crop species	Relevant biological characteristics of plant species	● Growth / Reproduction/ Ecological Interaction(s) / etc.
Characteristics of receiving environment	1) Relevant biological characteristics of release environment(s)	● Abiotic / biotic aspects
	2) Relevant (agronomic) management of NPBT-crop	● Management changes based on intended trait(s)

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This study addresses new plant breeding techniques (NPBTs) and, in particular, issues relevant for the assessment of potential risks associated with crops obtained through NPBTs. These issues are an emerging topic in ongoing biosafety discussions at European and global level.

The report provides an overview of different NPBT approaches and highlights specific aspects that are relevant when considering the potential adverse effects of NPBT-crops. The study investigates whether the current requirements for a risk assessment of genetically modified organisms contained e.g. in EU-regulations would provide an appropriate framework for addressing the potential risks associated with NPBT-crops. A set of criteria for the assessment of NPBT-crops is presented and open questions on the risk assessment of NPBT-crops are identified.