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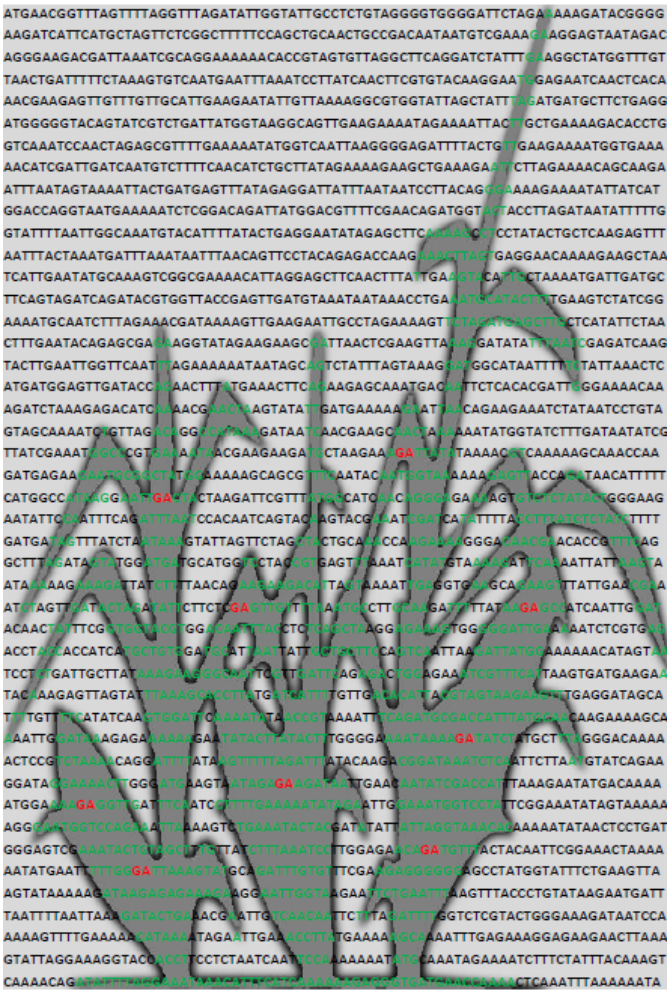
Biosafety Considerations for Plants developed by Genome Editing and other new Genetic Modification Techniques (nGMs) and Considerations for their Regulation



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Risk Assessment of Plants developed by new Genetic Modification Techniques (nGMs)

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Final report of the R&D project (FKZ: 3516 89 0400; lot1)

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Cover picture: Genome editing illustration: the collage shows the coding sequence of the cas9 gene of *Streptococcus gordonii* (*NCBI:txid467705*) in the background of a stylized corn plant with some color-highlighted sections symbolizing genome edited sequences (Artwork © M. Eckerstorfer).

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Abbreviations

AB	Accelerated breeding
AGES	Austrian Agency for Health and Food Safety Ltd.
AHAS/ALS	Acetolactate synthase
APHIS	Animal and Plant Health Inspection Service (US Department of Agriculture)
BfN	Bundesamt für Naturschutz
Cas	CRISPR-associated sequence (protein)
CENH3	Centromer histone3
CMS	Cytoplasmic male sterility
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DICER	Endoribonuclease/helicase with RNase motif facilitating RISC activation
DH	Double haploid
DR	Disease resistance
DSB	Double-strand break
dsRNA	Double-strand DNA
ECJ	Court of Justice of the European Union
EEA	European Economic Area
EFSA	European Food Safety Authority
EPSPS	5-enolpyrovylshikimat-3-phosphatsynthase
ERA	Environmental Risk Assessment
EU	European Union
EXZACT™	ZNF-based technology (Dow AgroSciences and Sangamo BioSciences)
FDA	Food and Drug Administration (USA)
GE	Genome editing
GFP	Green fluorescent protein
GM	Genetically modified
GMO	Genetically modified organism
GRAS	“generally recognized as safe”
HDR	Homology-directed recombination
HI	Haploid induction
HPPD	Hydroxyphenylpyruvate dioxygenase
HR	Herbicide resistant
IR	Insect resistant

MAGE	Multiplex automated genomic engineering
MN	Meganuclease
nGM	New/novel genetic modification techniques
NHEJ	Non-homologous end joining
ODM	Oligonucleotide-directed mutagenesis
OECD	Organisation for Economic Co-operation and Development
OSR	Oilseed rape
PAM	Protospacer adjacent motive
PAT	Phosphinothricin acetyl transferase
PPV	Plum pox virus
PTGS	Post transcriptional gene silencing
RA	Risk Assessment
RB	Reverse breeding
rDNA	recombinant DNA
RdDM	RNA dependent DNA methylation
RISC	RNA-induced silencing complex facilitating RNAi effect
RNAi	RNA-interference
RTDS	Rapid trait development system™
SAM	Scientific Advisory Mechanism of the European Commission
SDN	Site directed nuclease
siRNA	small interfering RNA
SU	Sulfonurea
TALEN	Transcription activator-like effector nuclease
TILLING	Targeting induced local lesions in genomes
TLP	Trait landing pad
TGS	Transcriptional gene silencing
VAGE	Virus-aided gene expression
VIGS	Virus-induced gene silencing
WGS	Whole genome sequencing
WT	Wildtype
ZFN	Zink-Finger-directed Nuclease

Zusammenfassung

Die Frage, ob Anwendungen von Methoden der neuen Gentechnik (nGM) in der Pflanzenentwicklung zu nicht vernachlässigbaren negativen Auswirkungen auf Umwelt und/oder Gesundheit führen können, ist für die Diskussion über die Risikobewertung dieser Methoden von eminenter Bedeutung. Der aktuelle Wissenstand betreffend die spezifischen Risiken dieser Anwendungen ist jedoch begrenzt, insbesondere für neu entwickelte nGMs, wie z.B. Genome Editing und ihren Variationen, wie z.B. Base Editing. Dies führt zu Unsicherheiten bei der Risiko-/Sicherheitsbewertung von Pflanzen, die mit den verschiedenen nGMs, darunter Genome Editing, Cisgenese, Transgrafting, Haploidinduktion oder Reverse Breeding entwickelt werden.

In einer Literaturrecherche wurden mittels nGMs entwickelte Pflanzen identifiziert, die für die zukünftige landwirtschaftliche Nutzung relevant sein können. Solche nGM Pflanzen wurden auf Risiken analysiert, die entweder (i) mit ihren entwickelten Eigenschaften und ihrer Verwendung oder (ii) mit unbeabsichtigten Veränderungen durch die nGMs oder andere während der Zucht angewandte Methoden verbunden sind.

Mehrere Merkmalsgruppen dürften in Zukunft für nGM Pflanzen besonders relevant werden, beispielsweise Herbizidresistenz, Resistenz gegen verschiedene Pflanzenpathogene sowie Anwendungen zur Veränderung der inhaltsstofflichen Zusammensetzung, der Morphologie und Fitness, wie z.B. erhöhte Resistenz gegen Kälte/Frost, Dürre oder Salzgehalt bzw. Pflanzen mit veränderten Reproduktionseigenschaften. Einige Merkmale, darunter Resistenzen gegen bestimmte Herbizide, sind bereits von existierenden konventionellen oder gentechnisch veränderten Pflanzen (GV Pflanzen) bekannt. Im Zuge der Risikoabschätzung die für derartige GV Pflanzen durchgeführt wurde, konnten eine Reihe von potentiell nachteiligen Effekten identifiziert werden und diesbezügliche Risiken charakterisiert werden, beispielsweise die Entwicklung von herbizidresistenten Unkräutern. Manche der in nGM Pflanzen entwickelten Merkmale sind hingegen neuartig, d.h. sie sind derzeit nicht in landwirtschaftlichen Pflanzen vorhanden und wurden noch nicht über längere Zeit in der landwirtschaftlichen Praxis angewendet. Bei manchen dieser nGM Pflanzen sind die den neuartigen Merkmalen zugrunde liegenden physiologischen Mechanismen noch nicht ausreichend aufgeklärt. Bestimmte Charakteristiken von Anwendungen des Genome Editing, wie das typischerweise geringe Ausmaß der dabei im Normalfall hervorgerufenen Sequenzänderungen im Pflanzengenom und die vergleichsweise höhere Präzision, können nicht als verlässlicher Hinweis im Hinblick auf ihre Sicherheit angesehen werden. Wesentlicher für ihre Bewertung ist, ob die mit neuen gentechnischen Methoden hergestellten Merkmale neuartig sind und ob die mit der jeweiligen Anwendung verbundenen Risiken auf Basis des vorhandenen Wissensstandes eingeschätzt werden können oder ob eine umfassende wissenschaftliche Risikoabschätzung dafür notwendig ist. Alle in dieser Arbeit analysierten nGM Techniken können in unterschiedlichem Ausmaß unbeabsichtigte molekulare Veränderungen in den modifizierten Pflanzen hervorrufen. Die höhere Geschwindigkeit der Pflanzenentwicklung bei Anwendung von nGM Methoden kann jedoch die Erkennung und Beseitigung unbeabsichtigter Veränderungen erschweren und dazu führen, dass diese unbemerkt bleiben. Daher ist es notwendig, auch für Pflanzen, die mit nGMs erzeugt wurden, eine fallspezifische Risikoabschätzung auf Basis der für bisherige GVOs entwickelten Prinzipien und Ansätze durchzuführen. Im Rahmen dieser Risikoabschätzung sollte auch eine geeignete molekulare Charakterisierung durchgeführt

werden, um unbeabsichtigte Veränderungen zu erkennen und/oder die Abwesenheit von unerwünschten transgenen Sequenzen zu bestätigen. In dieser Arbeit wird dargestellt, welche Überlegungen dazu angestellt werden sollten und vorgeschlagen, wie dabei vorgegangen werden sollte. Die wesentlichen Resultate der Studie sind auch in ECKERSTORFER et al. (2019b) veröffentlicht. Um allfällige Widersprüche zu vermeiden sind die entsprechenden Textpassagen identisch.

Summary

The question whether new genetic modification techniques (nGM) in plant development might result in non-negligible negative effects for the environment and/or health is significant for the discussion concerning their risk assessment. However, current knowledge to address this issue is limited for most nGMs, particularly for recently developed nGMs, like genome editing, and newly emerging variations of genome editing approaches, e.g. base editing. This leads to uncertainties regarding the risk/safety-status of plants which are developed with a broad range of different nGMs, especially genome editing, and other nGMs such as cisgenesis, transgrafting, haploid induction or reverse breeding.

A literature survey was conducted to identify plants developed by nGMs which might be relevant for future agricultural use. Such nGM plants were analysed for potential risk issues associated either (i) with the traits developed with nGMs and the intended use of the nGM plants or (ii) with unintended changes resulting from the nGMs or other methods applied during breeding.

In the future several traits are likely to become particularly relevant for nGM plants, namely herbicide resistance (HR), resistance to different plant pathogens as well as modified composition, morphology, fitness (e.g. increased resistance to cold/frost, drought, or salinity) or modified reproductive characteristics. Some traits such as resistance to certain herbicides are already known from existing conventional or GM crops. The previous assessment of such GM crops identified specific issues of concern and/or risks, such as the development of herbicide resistant weeds. Other traits contained in nGM plants are novel; meaning they are not present in agricultural plants which are currently cultivated. For some of these traits the underlying physiological mechanisms are not yet sufficiently elucidated. Characteristics of some genome editing applications, e.g. the small extent of genomic sequence change and their higher targeting efficiency, i.e. precision, cannot be considered an indication of safety per se, especially in relation to the novel traits created by such modifications. All nGMs considered in this study can result in unintended changes of different types and frequencies. However, the rapid development of nGM plants can compromise the detection and elimination of unintended molecular changes and the associated effects. Thus it is required to conduct a case-specific premarket risk assessment for nGM plants following the principles and approaches for the previous GMO, including an appropriate molecular characterization to identify unintended changes and/or confirm the absence of unwanted transgenic sequences. The study proposes a stepwise approach how such an assessment should be conducted and outlines which issues should be considered for the assessment. The main results of the study are published in ECKERSTORFER et al. (2019b). To avoid misunderstandings the respective text parts are identical.

1 Introduction

In the general public the biotechnological modification of crop plants is largely associated with the application of genetic engineering, or GM technology. In recent years a broad range of “new genetic modification techniques” (nGMs) was additionally developed for the (genetic) modification of organisms, including plants, for research purposes or for the development of crops for agricultural use. These nGMs are also referred to as “new techniques” or “new breeding techniques” in other publications (LUSSER et al. 2012, SAM 2017, VOGEL 2016). However, the individual methods which are referred to by this term are quite different in nature and some methods are not any “newer” than GM technology. To avoid possible misconceptions that these technologies are just variants of conventional cross-breeding methods we do not use these terms in this report. Typically the final products developed by application of nGMs differ from classic GMOs, i.e. organisms, which contain heritable recombinant genetic constructs assembled from a number of genetic elements typically derived from different species.

Whether and which of these nGM applications are or should be regulated according to Directive 2001/18/EC at the EU-level and by national regulations implementing that Directive such as the German Gene Technology Law (1990), was debated intensely over the last years. A decision by the European Court of Justice clarified that nGM plants developed by means of genome editing approaches are subject to regulation under Directive 2001/18/EC (ECJ 2018). Such decisions are connected to important requirements, e.g. concerning the assessment of regulated products for biosafety: Applications subject to Directive 2001/18/EC and the national GMO regulations of EU Member States need to undergo a risk assessment prior to authorisation, which is addressing risks to human and animal health and the environment due to the deliberate release of these GMOs into the environment and their placing on the market. This risk assessment needs to be conducted on a case-by-case basis since the knowledge as regards the effects of the genetic modifications on the overall organism is limited and the interactions of GMOs with their environment are complex (cf. EKAH 2012). According to an analysis by the Swiss Ethics Committee on Non-Human Biotechnology – ECNH (EKAH 2016) similar factors need to be considered for all the different nGMs. The findings of the ECNH indicate that for most nGMs the knowledge base for a proper understanding of the biological mechanisms underlying these techniques as well as their effects is still inadequate.

It is therefore relevant to determine which risk issues of specific nGM applications need to be considered and to develop suitable approaches for the risk assessment (RA) of such applications and their products. However, until now only few studies were conducted to address these questions specifically and many necessary details are still not addressed sufficiently.

The situation is also complicated by the fast technical development in the field of nGMs and the recent availability of improved and/or technologically advanced methodologies. The approaches for RA which are used to evaluate the effects of such products should be appropriate to address the relevant characteristics of newly developed nGM approaches and in particular of the nGMs, which are primarily used for the development of agricultural products, such as genome editing. The challenge is that RA procedures need to be flexible enough so that they can be applied to the whole range of different nGM applications including any newly developed nGM approaches. In addition some newly developed nGMs

are challenging the currently used RA and will require the development of adapted approaches for RA.

The development of CRISPR/Cas technology for genome editing, i.e. the targeted introduction of changes to specific genomic sequences, might be taken as an example. Only available for some years, this technology is universally regarded to be a game changer as far as versatility, speed and technical ease of applicability is considered. In addition this technique is also a good example for the rapid and widespread adoption of a specific methodology in scientific research as well as in applied research and product development. Further development of CRISPR/Cas technology led to new concepts of application, including new approaches to the modification of the epigenetic regulation of gene expression, i.e. epigenetic engineering, or the design of transgenetic constructs known as gene drives, which are preferentially passed on during sexual reproduction and therefore are able to spread through the genome of entire populations rapidly. Both types of applications need to be addressed by specifically designed RA approaches taking into consideration the respective characteristics of such approaches.

One objective of the study at hands is to describe the technologies used for the development of new crop varieties and to provide indications, which types of new products may be developed with these technologies in the foreseeable future. Such information is needed for the development of approaches to regulation and risk assessment which are fit for the purpose, i.e. best suited to the technical challenges and able to ensure an appropriate margin of safety with the resources available for risk assessment.

Building on this information a further objective of the study is to analyse whether specific nGMs might be associated with a potential for specific adverse effects and how such effects might be evaluated during RA. Defining the specific biosafety issues associated with nGM products is an important issue in the general discussion on nGMs at the political level. At any rate further efforts to addressing biosafety-related questions are needed to support the ongoing discussion process concerning the agricultural application of nGM plants.

2 nGMs addressed in this study

As outlined in the introduction there is no universally accepted definition for nGMs and thus no defined spectrum of nGMs. The number of techniques which are included in the group of nGMs was rising in the recent years due to new developments of technology and new applications of certain nGMs, cf. (SAM 2017). A common starting point is the range of nGMs included in the deliberations of the EU working group on new techniques (NTWG 2011) and the accompanying study undertaken by the Joint Research Centre of the European Commission on the subject (LUSSER et al. 2011a). Subsequent studies addressed a different, sometimes extended range of techniques, one of the most comprehensive overviews was provided by an update to a Swiss study on nGMs (VOGEL 2016).

For the purpose of this study the list of nGMs in Table 1 is used to capture the different approaches employed by the different techniques.

The nGMs, which will be discussed in this study, are grouped into several categories clustering together nGMs, which share a specific goal of application and/or exploit similar cellular mechanisms. An example for such a category are nGMs, which are using different types of nucleases to introduce genomic changes at specific genomic loci, e.g. for introducing mutations in a targeted way at specific genomic sequences (see Table 1 and Chapter 2.1 below for further information).

Likewise other nGMs which share similar objectives are grouped together in own categories. A number of nGMs which will be discussed in this study are listed without categorisation.

Note should be taken that no universally accepted terminology is available for many of the nGMs and a number of synonymous terms are used to describe them in the literature. For this study we will use the terms introduced in Table 1. An overview on other terms which are used synonymously is presented e.g. in VOGEL (2016).

This chapter is providing a general introduction to the nGM techniques addressed in this study, focusing on nGMs which can be clustered into different categories based on the physiological mechanisms which are exploited and the nature of the intended modifications. Three of these broader categories are introduced: genome editing approaches based on different site directed nucleases (SDN), genome editing using oligonucleotide templates to induce specific mutagenesis events (ODM), and approaches directed to the modification of gene expression based on different mechanisms, e.g. overexpression of specific genes, silencing of gene expression or the modification of epigenetic control of gene expression.

The other nGMs, which are not falling into these categories, are briefly introduced and described in respective subchapters of Chapter 4.

Table 1: Overview on the nGMs addressed in this study

Category	nGM	Specific nGM approach	See Chapter
Genome editing with Site Directed Nucleases (SDN)	CRISPR-based systems for genome editing	SDN-1 SDN-2 SDN-3 Base editing	4.1
	TALE-directed nuclease systems (TALEN)	SDN-1 SDN-2 SDN-3	4.2
	Zinc-Finger-directed nuclease systems (ZFN)	SDN-1 SDN-2 SDN-3	4.3
	Meganuclease-based systems (MN)	SDN-1 SDN-2 SDN-3	4.4
Genome editing by oligonucleotides	Oligonucleotide directed Mutagenesis (ODM)	-	4.5
	Multiplex Automated Genomic Engineering (MAGE)	-	4.6
Modification of gene expression	RNA dependent DNA Methylation (RdDM)	-	4.7
	Other techniques for the modification of gene expression	Virus-aided gene expression (VAGE) RNAi-based gene silencing CRISPR-based modification of gene expression	4.8
	Cisgenesis/Intragenesis	Cisgenesis Intragenesis	4.9
	Transgrafting	Grafting on GM Rootstock	4.10
	Agroinfiltration	Agroinfiltration Agroinfection Floral Dip	4.11
	Haploid Induction (HI)	CenH3-based HI	4.12
	Reverse Breeding	-	4.13

2.1 Genome editing with Site Directed Nucleases (SDNs)

This category comprises a number of techniques, which are aimed at a similar objective: the modification of genomic DNA of crop plants at specific target sequences by means of nucleases, which introduce single-strand breaks (“nicks”) or double-strand breaks (DSBs) in the genomic DNA at specific locations. Several approaches have been developed over the

last years to achieve this goal, employing a set of different site-directed nucleases: In particular CRISPR-based nucleases, TALE-nucleases, Zinc-Finger-nucleases and meganucleases are used

Different types of intended outcomes can be achieved during genome editing, depending on the specific cellular mechanisms which are triggered by these nucleases and depending on the presence of additional genetic elements to instruct specific genomic changes. According to LUSSER et al. (2011a) and PODEVIN et al. (2013) three of these classes, i.e. SDN-1, SDN-2 and SDN-3, are distinguished, which are described further below. All types of results are due to the action of DNA repair processes operating in the modified plant cells, which are activated by breaks in the cellular DNA to fix such breaks and preserve genome integrity. An overview on the involved mechanisms of non-homologous end joining (NHEJ) and homologous recombination is given in PODEVIN et al. (2013). A number of different cellular proteins participate in these mechanisms, which recognize DNA breakpoints, process the ends of the DNA exposed strands, and finally connect (i.e. ligate) the processed DNA ends. Particularly during NHEJ the original sequence present at the recognition sites for the particular SDNs may be changed by these processes – introducing various types of changes to the genomic DNA sequences at or around the recognition sequence (VOYTAS 2013).

A new approach to site directed genome editing, referred to as “base editing”, was developed in the recent years. This approach is based on the targeted chemical modification of specific base moieties in the intact genomic DNA strand, rather than on introducing sequence specific single- or double-strand breaks into genomic DNA (KOMOR et al. 2016, REES&LIU 2018).

SDN-1 type approaches are based on small sized random sequence changes at and around the double-strand break points due to NHEJ by the error prone mechanisms of canonical end joining or alternative end-joining pathways (PODEVIN et al. 2013). In a significant proportion of repair events the original sequence is not regenerated, but typically small deletions or less frequently point mutations or insertions are introduced (VOYTAS 2013). If the sites of DSBs are within the coding regions of genes this can result e.g. in the disruption of open reading frames and the knock-out of the respective gene function or the introduction of sequence changes in the targeted genes, which result in the expression of products with modified properties. One example for the latter outcome is the use of SDN-1 approaches for the development of crop plants with herbicide resistance traits. The resistance to certain herbicides is resulting from the introduction of site-specific mutations into endogenous plant genes, whose products are inhibited by the respective herbicides in wildtype plants (LUSSER et al. 2011a) (PODEVIN et al. 2013).

SDN-2 applications are aimed to introduce specific sequence changes to the DNA region at the site of SDN-generated DSBs. To specify the intended DNA sequence changes, additional small DNA fragments which are highly homologous but not identical to the target DNA are supplied in *trans* to the repair machinery as donor or repair templates. The sequence changes are then introduced mostly via the homologous recombination pathway of repair and are typically aimed to correct mutations occurring in a specific crop genome or to introduce new intended (point) mutations (LUSSER et al. 2011a) (PODEVIN et al. 2013).

SDN-3 applications also use donor DNA fragments, which are supplied in addition to a site-specific nuclease to the targeted cells. However SDN-3 templates typically contain additional

sequences which are unrelated to the DNA at the site of repair between flanking sequence elements homologous to the genomic sequences at the targeted location. Insertion of the template sequences via homologous recombination repair can give rise to targeted gene insertion or targeted gene replacement (VOYTAS 2013). If internal gene sequences are deleted in the template DNA, insertion of these donor sequences are directing the respective disruption of endogenous gene copies and typically knock out the respective gene function(s).

Targeted deletions in genomic sequences can be achieved by SDN-3 approaches, i.e. throught the insertion ofgene copies which carry a deletion in order to disruption endogenous genes; this may also be facilitated by an adaptation of the SDN-1 approach as outlined above. To achieve the construction of genomic deletions two SDNs are used simultaneously which introduce two DSBs at different target sequences in a certain genomic region, flanking the genomic sequences which should be deleted. It is important to note that this approach should not be confused with multiplexed SDN approaches, which are conducted to simultaneously change unrelated genomic sequences at non-neighbouring genomic locations (cf. VOGEL 2016).

Base editing uses modified SDNs, which retain the ability to target specific DNA recognition sequences, coupled to enzyme domains, e.g. cytidine-deaminase moieties, which can change the targeted DNA sequence via the chemical modification of specific nucleobases at the target site (KOMOR et al. 2016). According to the specific function of the editing enzyme used for base editing, the range of mutations which can be created at the target site is restricted to modification of a single type of nucleobase, cytidine. Thus base editing approaches directly initiate specific sequence changes (typically C to T or A to G) at the targeted genomic locations, rather than leading indirectly to sequence changes by inserting DNA breaks at the targeted sequences and the subsequent generation of mutated sequences by cellular DNA-repair mechanisms (MATSOUKAS 2018). Base editing therefore is meant to avoid unintended sequence changes, like random insertions or deletions, which typically can occur with SDN approaches based on repair of DSBs at the targeted genomic locations.

2.2 Genome editing by oligonucleotides

This category of genome editing approaches is distinguished from the above described SDN approaches since it does not employ site-directed nucleases to initiate intracellular reactions leading to genomic sequence change at specific locations, but rather uses synthetic oligonucleotides which are introduced into specific target cells for directed mutagenesis of genomic DNA. In comparison to SDN applications Oligonucleotide Directed Mutagenesis (ODM) is using a mutagenic donor/template oligonucleotide alone without the addition of any site-directed nuclease (RIVERA-TORRES&KMIEC 2016).

Multiplex Automated Genomic Engineering (MAGE) is a development of an advanced method to achieve highly multiplexed GE in bacteria using sets of oligonucleotides with degenerate sequences to target multiple genomic targets (WANG&CHURCH 2011).

2.3 Modification of gene expression

This category of applications is distinguished from other nGMs as the objective of these approaches is to modify the expression of specific genes without changing the DNA sequence of the respective targeted expression units. It can be done by a variety of approaches directed to influence transcription or translation of the respective plant genes, by silencing genomic repressor sequences using epigenetic modifications or increasing transcriptional strength by targeted localisation of heterologous transcriptional activators.

RNA dependent DNA Methylation (RdDM) is a method of epigenetic engineering to deliberately modify the genomic patterns of methylation of specific target genes or their regulatory sequences. The method can be used for both transcriptional gene silencing (TGS) if the targeted sequences are elements activating the transcription of the genes of interest (e.g. promoter or enhancer elements), or to increase production of certain gene products if the genomic target sequences function to repress the expression of endogenous plant genes.

RNAi based gene silencing is a widely used method for interfering with the expression of endogenous genes by post-transcriptional gene silencing (PTGS).

Virus-aided gene expression (VAGE) is a technique that aims to increase the amount of specific gene products in plant cells by using virus-based vectors for transiently introducing expression constructs into the target cells (VOGEL 2016), e.g. for increasing the number of expressed copies of a target gene. A related technique is virus-induced gene silencing (VIGS) aimed to introduce recombinant constructs to reduce or prevent the expression of endogenous plant genes, i.e. to “silence” these targeted genes.

CRISPR-based modification of gene expression is a new concept using the sequence-specific DNA-binding domain of CRISPR/Cas proteins to target effectors of gene expression to specific genomic loci (VOGEL 2016). For these applications CRISPR/Cas-complexes with disabled nuclease domains are employed, which by themselves can act as transcriptional regulators due to their ability to block the binding of endogenous transcription factors to their regulatory sequences (GUHA et al. 2017). To elicit specific effects heterologous protein domains, which specifically influence gene expression at the respective loci, may be fused to the inactivated nuclease domains. These regulators can either represent transcriptional activators or repressors or act by modifying the patterns of epigenetic regulation at the target loci, e.g. by adding or erasing epigenetic signals at the respective genomic DNA (methylation of nucleotides) or histones (methylation or acetylation of histone proteins) associated with the targeted genomic loci (GUHA et al. 2017), (KOMOR et al. 2017).

3 Approach for the characterisation of individual nGMs

Chapter 4 provides a description of the individual nGMs listed in Table 1. This characterisation is addressing a number of general features of these individual nGMs, according to the following list:

- Outline of concept and general characteristics of a specific nGM
- Experiences with and applications generated by an nGM
- Possibilities for combination of specific nGMs with other approaches
- Possibilities for detection and identification of products generated by an nGM

In the following a brief outline on the information used to describe the above listed general features is provided.

3.1 Outline of concept and general characteristics of specific nGMs

This section presents a brief outline of the objective to apply a specific nGM and explain which outcome should be achieved by a certain nGM. In case an nGM may be used in different ways, the respective variants concerning the application of an nGM will be introduced. If more than one concept for application of a particular nGM exists, the differences between these concepts for application of an nGM are outlined.

The information is mainly based on available reviews on the respective nGMs with a particular emphasis on recent reviews as far as current developments are concerned.

3.2 Experiences with nGMs and applications generated by nGMs

This section addresses three aspects which are considered relevant for considerations of experience with the various approaches and their history of use.

Firstly, information on early applications of certain techniques were retrieved from available reviews addressing the particular nGMs (e.g. (LUSSER et al. 2011a) (VOGEL 2012). This hints at the amount of experience with the respective techniques, in particular as application in research activities with relevant crop species and crop development are concerned.

Secondly the available scientific literature concerning the application of the nGMs addressed in this study was screened, in particular the most recent literature, which was not already reviewed in previous studies addressing the respective nGMs. The retrieved publications provide an overview on documented examples of application of the different nGMs, as regards the targeted plant species, the used methodological approaches and the purpose of the research, and if of specific developments are described the traits which are targeted by the specific nGM approach. Details concerning the search strategy used to retrieve relevant publications are further described in Chapter 3.5.

Information on product development for commercialisation focuses on late stages of development (field trials) and products intended for commercialisation or already commercialised in non-EU countries was collected from a number of sources, including published reviews and information on inquiries related to commercialisation of specific products available from relevant countries, which are publicly available. Specifically

information on inquiries to USDA-APHIS concerning the regulatory status of certain developments was taken into account.

3.3 Combination of nGMs with other approaches

In most cases single nGMs are not used in isolation, but in combination with other methods, including e.g. conventional breeding steps, in vitro cultivation of plant cells and tissues, and the application of GM technology and other nGM methods.

Such additional methods are used to facilitate the overall development strategy of a specific nGM crop; their implementation provides relevant benefits as regards e.g. the time needed for development, the ease of development of a specific nGM product or other aspects relevant for the process of development or for the characteristics of the nGM product. A number of nGM approaches are used in this way as technical tools e.g. to express traits which are needed to enable the development of an nGM product from a different nGM category. For example nGM approaches (like genome editing or RNAi) can be used to knock out or silence specific endogenous plant genes to e.g. initiate early flowering as a tool for developing products by accelerated breeding, or to suppress meiotic recombination in plants which are used in reverse breeding applications. Another example is the use of transgrafting to produce plant progenies with intentionally modified patterns of epigenetic regulation by means of RNA dependent DNA methylation (RdDM). In such an approach small RNAs, which are expressed in a GM rootstock and disseminated throughout the whole plant into the non-GM parts, i.e. the scion, are used to facilitate the production of epigenetically modified, non-GM offspring.

The additional use of specific (biotechnological) methods might be required to enable the successful application of a specific nGM approach in a number of cases. As an example the use of protoplast culture is regarded an essential requirement for genome editing applications, which are based on the direct introduction of the functional nuclease into recipient plant cells. Such approaches are currently considered as an alternative to methods which involve the transient or stable GM modification of the recipient plants to enable the expression of transgenic constructs coding for the components of a specific nuclease system for genome editing in the transformed cells.

For some of these combinations alternative approaches may exist. However, such alternatives may have their own disadvantages, e.g. as regards their association with other potential (adverse) effects which need to be considered for risk assessment.

For purposes of an overall evaluation of the risk associated with plants generated by certain nGMs the (potential) effects of all methods, which are involved in the generation of a specific nGM plant, need to be considered.

According to information concerning a combination of new techniques derived from prior reviews, e.g. VOGEL (2012) and AGES (2013), as well as from the conducted literature survey examples for combinations of different nGMs are listed in subsequent chapters addressing this issue for the discussed types of nGM methods.

3.4 Detection and identification

The issues of detection and identification of products generated by the application of nGMs are important in the overall discussion on the subjects of regulation and risk assessment of such technologies. This was highlighted in public debate as well as in relevant reports, e.g. in the explanatory note on New Techniques in Agricultural Biotechnology by the High Level Group of Scientific Advisors to the European Commission (SAM 2017).

However as noted by VOGEL (2012) a clear distinction has to be made between the two issues:

- Detection, which is defined as the ability to determine whether a specific change in the genetic material is occurring in a particular crop and
- Identification, defined as the ability to determine that the detected product relates to a specific nGM application, i.e. to determine that the specific genetic modification was introduced by a certain developer using a specific nGM approach.

A number of different points of discussion are relevant in the framework of the ongoing discussion:

- What are the objective(s) for detection and identification strategies for nGM applications?
- Which kind of prior knowledge is needed for detection and/or identification and which information would be available for nGM products in different scenarios (e.g. for regulated/unregulated products, products which originate from the EU market/imported products from other countries with different legislations)?
- Which methods are available and appropriate for the analytical detection of different types of nGM crops and which challenges are associated with their implementation?
- Which information can be generated by analytical methods and/or alternative approaches to ensure traceability and how is this kind of information supporting the aspired objectives?

3.4.1 Objectives for detection and identification

One of the goals of the existing EU regulations for GMOs is to ensure the traceability of GMO products on the market, including GM food and feed products, and to protect the right to freedom of choice for consumers (FRAITURE et al. 2015). Regulation (EC) No 1829/2003 foresees as a requirement for authorisation that an event-specific detection method is proposed by the applicant, which is validated by the European Union Reference Laboratory prior to authorisation. The validated method can then be used in the enforcement laboratories of EU Member States for food and feed control. For nGM applications which are covered by the existing EU regulatory framework for GMOs, including applications of genome editing according to the respective ruling of the European Court of Justice (ECJ 2018), the developers of such applications need to provide appropriate information on the specific products to support detection and identification.

3.4.2 Prior knowledge for detection and/or identification

An important consideration concerning detection and identification of nGM crops is the availability of appropriate prior information on the molecular modifications present in a

specific nGM crop or product. Specifically the ability to identify a particular nGM product is compromised if no such prior information is available. A lack of such information is expected for unauthorised GM or nGM products entering the EU market.

Unauthorised GM products may occur on the market due to asynchronous approval in different legislations, upon expiry of prior authorisations or through accidental release of GMOs during development or field testing (FRAITURE et al. 2017, LI et al. 2017d). The detection and identification of unauthorised GM products of the latter category is posing a number of serious challenges due to limited or lacking information on the specific transgenic modifications present in these products and the unavailability of event-specific detection methods (FRAITURE et al. 2017).

For patented applications prior knowledge on the molecular features of unauthorised GM or nGM products may be available. Such features can then be directly targeted by detection strategies.

3.4.3 **Methods for the analytical detection of nGM crops**

Current discussions regarding new strategies to detect and identify GMOs and in particular unauthorised GMOs are also relevant for nGM crops, e.g. as the available methods, their limitations and information requirements for their application are concerned. The current standard for detection and identification of authorised GM products are event-specific real-time quantitative PCR methods, however a variety of different methods is available or in development for both molecular characterisation of GM crops (LI et al. 2017d) and GMO detection (FRAITURE et al. 2015). Some of them are particularly suited for the rapid detection of specific transgenic proteins in environmental samples, other methods, which are targeting transgenic elements common to many GMOs, are used to screen whether any of these GMOs occur in certain products, and a variety of other methods are developed to facilitate multiplex detection strategies, i.e. methods which can simultaneously detect several different GMOs in a single test setup (FRAITURE et al. 2015). In case no adequate prior information on the specific molecular changes incorporated into particular nGM products is available, untargeted methods need to be applied to screen the whole genome of a specific plant for molecular features which are indicative for a specific nGM application. Similar approaches as used for characterisation and detection of unauthorised GM crops with transgenic inserts of unknown sequence could be applied (FRAITURE et al. 2017, LI et al. 2017d). Detection approaches based on whole genome sequencing in particular may be feasible if genomic information like a suitable reference genome for the crop species is available (SAM 2017). However, identification may not be readily achieved in such cases (FRAITURE et al. 2016, SAM 2017).

In addition to considerations regarding the available knowledge on nGM applications, the specific characteristics of the different nGM approaches must be taken into account for the discussion concerning detection and identification of the respective products. Particularly the type of modification introduced by an nGM method and the size, number and nature of genomic modifications and their presence in nGM plants is important for the detection and the unambiguous identification of a specific nGM application.

In relation to the overall genome size of the respective species and the extent of genomic variation in that species a minimum number of sequence changes is necessary to differentiate modifications introduced by specific nGM applications with a sufficient certainty

from changes due to other influences, e.g. spontaneously occurring mutations. The smaller the modification the greater is the methodological challenge, particularly without any prior information on the nature of the modification. Some authors claim that intended changes with a size of a few or even to a single base pairs as generated with certain genome editing approaches (e.g. SDN-1 and SDN-2, ODM) may still be detected with a minimum of prior information available (FRAITURE et al. 2016). However, a number of factors which negatively influence the possibility to detect such changes by whole genome sequencing (WGS) approaches need to be considered (WILLEMS et al. 2016):

- availability of a suitable reference genome for the species in question
- technical challenges related to the different sequencing approaches, technical platforms and bioinformatics methods used to generate and assemble sequence data with an appropriate quality to reliably identify small sequence changes
- challenges related to the genomic characteristics of the plant species which is subjected to modifications introduced by nGMs, e.g. genome size, extent of genetic variability within varieties which are used in agricultural production, level of knowledge as regards functional annotation of sequence data

Whereas detection of specific small sized genomic changes might be difficult, the identification of a specific nGM product with only a few or a single base pairs change(s) may be impossible to achieve. Only when the number of changes at a specific genomic locus or at genetically tightly linked loci is sufficiently high, robust analytical results from WGS approaches may allow for both detection and identification.

Other nGM applications result in larger sized genomic modifications of a kind, which would not readily arise by spontaneous genetic variation or approaches leading to random mutagenesis. Such products are generated e.g. in the course of cisgenesis, intragenesis and genome editing approaches, which are aimed at the integration of larger genetic elements previously not present in the species of interest (i.e. SDN 3 applications). Similar approaches to detection and identification as currently applied for GMOs may be used for such products (SAM 2017). Also transgenic sequences which are used in transgrafting and agroinfiltration may be detected by such approaches, if the appropriate sample material is analysed, i.e. material from modified plant parts or tissues, which are exposed to transgenic Agrobacteria and contain the transiently present transgenic constructs.

In contrast nGM products generated by applications which do not result in DNA sequence changes in the targeted test materials cannot be detected and identified by the methods referred to above. This is relevant for such nGM applications which do not aim at genetic modifications, like RdDM (if no further transgenic modifications are present in the tested product), and nGM approaches, where relevant genetic modifications are only present at intermediate stages (e.g. for Reverse Breeding or other applications directed to establish non-GM modified lines by segregation at downstream breeding steps) or in plant parts other than the product subjected to analytical testing (non-modified plant parts and tissues from transgrafting and agroinfiltration applications). Detection in such cases can only be based on an assessment of distinguishing phenotypic or biochemical characteristics. For detection of specific compositional characteristics OMICs approaches may be used, however the application of such tools is not common or routine currently, in particular for cases where no prior information is available and untargeted OMICs approaches would be needed. Targeted

OMICs analyses in comparison are easier to be implemented, however their use is only possible based on appropriate prior information on the characteristic molecular changes that are present in a certain nGM product.

3.4.4 **Further challenges for analytical detection**

Firstly most analytical samples do not contain pure material composed of 100% of a specific nGM product, e.g. in routine analytical practice, samples typically contain mixtures of products from different origin. Therefore nGM products may only be present at medium or low levels or even at trace amounts. If analytical methods can be applied for nGM products which combine high sensitivity for specific genetic modifications with a good ability for quantification, like the PCR-based detection methods currently used as a gold standard for GMOs, this might not be a particular problem. However, mixed samples may present technical challenges for other approaches, specifically those based on untargeted WGS to identify genomic modifications (FRAITURE et al. 2017, WILLEMS et al. 2016).

Initial considerations for a statistical framework for WGS detection methods addressing these challenges have been developed (WILLEMS et al. 2016) and calculations were provided for a number of crop species with different genome sizes regarding the amount of sequence data needed for detection or identification of GMOs by WGS methods. The conclusions of this study were also evaluated for validity based on data generated for a mixture of different maize lines as reviewed in a recent publication (FRAITURE et al. 2017).

While detection may still be achieved in samples of mixtures containing lower amounts of targeted ingredients, the quantity of sequence data which is necessary for detection, the associated technical difficulties, e.g. concerning the bioinformatics analysis of the sequencing data and the resulting costs of analysis are substantially higher than for pure products. Also the necessary technologies are not yet available at all enforcement laboratories in the EU Member States (FRAITURE et al. 2017). Depending on the crop species in question the WGS approach was however concluded to be a reasonable option as regards the detection of unapproved GMOs and possibly of crops generated by nGM approaches (FRAITURE et al. 2017)

Secondly the WGS approach may not be able to provide an unambiguous identification of a certain nGM product containing only a limited number of small sized genetic changes, e.g. introduced via targeted mutagenesis, even if the genomic modifications themselves can be detected by the analysis (FRAITURE et al. 2017). Depending on the specific situation the data might only provide an indication of the probability that a specific nGM product is contained. Unambiguous identification would then only be possible if further information on the origin of the sample is available or additional data, e.g. regarding the presence of further genetic signatures which are tightly linked to the genomic modification introduced by application of an nGM. In addition emerging methods to detect metabolic signatures, which are specific to certain nGM products, developed e.g. by genome editing approaches, may be used to establish data which can support the identification of such products (KUMAR et al. 2017).

The difficulties associated with the detection and identification have led to various efforts in different countries to develop new methods aimed at identifying direct or indirect signatures of nGM applications, specifically genome editing in various organisms (cf. IARPA 2017). The respective activities are on the one hand targeted to investigate the tools or methods which are currently available to detect modifications introduced by genome editing and the resulting metabolic changes and to

develop new approaches to both detection of the respective genetic and metabolic signatures for monitoring and enforcement. On the other hand the challenges associated with identification of nGM application are targeted, as well as the requirements to implement newly developed methods in practical terms, e.g. regarding evaluation, validation, establishing appropriate capabilities (infrastructure and human capacity).

3.5 Survey of relevant scientific literature

The literature search conducted in this study should identify publications from recent years which were not already covered in prior reviews addressing a range of nGMs relevant to this study (LUSSER et al. 2011a) (VOGEL 2012), or individual nGMs included in this study (e.g. CRISPR/Cas covered in (HILSCHER et al. 2017)). The general timeframe for the literature search included the period 2011 until June 2017, except as indicated below for some individual nGMs which were covered more recently by comprehensive reviews. The search was limited to articles published in English.

Scientific literature addressing the following list of nGMs was searched using the databases Scopus and ProQuest Natural Science Collection, which corresponds to the former Cambridge Scientific Abstracts database. It was conducted between May and June 2017. The following keywords and keyword combinations were used:

- TALEN: ("transcription activated-like nuclease*" OR TALEN OR "transcription activator-like effector nuclease*")
- Meganuclease: (meganuclease* OR "homing endonuclease*")
- Cisgenesis/Intragenesis: (cisgen* OR intragen* OR "all native DNA transformation" OR "all-native DNA transformation")
- ODM: (oligonucleotid* OR "oligonucleotide directed mutagenesis" OR ODM OR "chimeric oligonucleotid*" OR "chimeric RNA/DNA oligonucleotid*" OR chimeraplasty OR "site-directed mutagenesis" OR "gene targeting")
- Reverse breeding: ("reverse breeding" OR "crossover control")
- Haploid induction: (haploids OR CENH3 OR "haploid induction" OR "genome elimination")
- Agroinfiltration: (agroinfiltr* OR agroinocul* OR agroinfect*)
- MAGE: ("multiplex automated genomic engineering")

The keyword search was limited to 'title' and 'abstracts' and the keywords were complemented by the terms 'crop*' and 'plant*'. In addition various filters provided by the search mask of the respective search engines were applied in order to focus the search query on the use of the various nGMs in agricultural biotechnology and on peer-reviewed scientific articles. The aim was to exclude hits which apply to the use of the various nGMs in animals or for pharmaceutical or medical purposes.

The retrieved references were combined and duplicates were eliminated. Title and abstract of the remaining references were screened manually for fulfilling the intended search criteria.

Scientific literature for the remaining nGMs was retrieved using the Web of Science and pubmed databases. The following keywords and keyword combinations were used:

- CRISPR: ((plant OR plants OR plant* OR “plant breeding” OR crop* OR tree*) AND (crispr OR cpf1)) and ((crispr OR cpf1) AND tree* NOT (plant OR plants OR plant* OR “plant breeding” OR crop*)). Results were combined and screened for research papers and reviews published in the years 2016 and 2017.
- ZFN: (("zinc finger nuclease" OR ZFN) AND (plant* OR crop*))
- RdDM: =((TGS) AND (plant* OR crop*)) and ((RDDM OR RNA*directed DNA methylation) AND (plant* OR crop*))
- Transgrafting: (graft* AND (transg* OR transform* OR GM graft OR GM scion) AND (plant* OR crop* OR tree*)). The search term ((graft* OR transgraft* OR trans-graft*) AND ("GM rootstock*" OR "transgen* rootstock")) was used for transgrafting applications involving GM rootstocks. The search was focused on the years 2015 to 2017 to add to available literature.

Similar as indicated above the retrieved references were combined, duplicates were eliminated and the titles and abstracts of the remaining references were screened manually for relevant scientific papers or reviews.

4 Characterisation of individual nGMs

4.1 CRISPR-based systems for genome editing

4.1.1 Concept of CRISPR-based systems for genome editing

CRISPR-based systems for genome editing are RNA protein complexes, developed from bacterial nucleases, which were found to act as an adaptive immunity system against foreign DNA entering bacterial cells, e.g. phage DNA or plasmid DNA. Such CRISPR nucleases, like the Cas9 nuclease, are able to recognise and bind to specific DNA sequences in foreign DNA and can inactivate such DNAs by cutting both strands of the respective DNA fragments (see e.g. (AGAPITO-TENFEN&WIKMARK 2015)).

According to their ability to target specific DNA sequences and to introduce DSBs into specific sequences occurring in the targeted DNA such RNA-directed nucleases can also be applied for the targeted editing of crop plant genomes. The respective CRISPR-based nucleases are guided to a particular plant sequence by guide RNAs (gRNAs), which are designed to mimic the respective features for DNA recognition and nuclease attachment provided by the CRISPR-RNA (crRNA) and transactivating crRNA (tracrRNA) components of the original bacterial nucleases (PUCHTA&FAUSER 2014). CRISPR Type II systems, like CRISPR/Cas9 are used as a genome editing tool, since they can be programmed to recognise specific sequences with a single appropriate gRNA molecule as the only requirement in addition to the Cas9 nuclease protein (JINEK et al. 2012).

The versatility of the CRISPR-based systems for genome editing is based on the fact that it can target a wide variety of sequences in different genomes. To adapt the system to different genomes, just specifically designed gRNAs need to be provided in *trans*, without the need to redesign the nuclease component, e.g. the Cas9 nuclease.

A multitude of CRISPR-based genome editing tools have been developed, among them a number of specific nucleases, which contain other CRISPR nucleases than the initially used SpCas9 nuclease protein from *Streptococcus pyrogenes,* or modified Cas9-proteins which do not function as a nuclease but have other functional properties. The different systems have specific characteristics as regards precision, efficacy, intended or unintended effects and therefore need to be considered separately for the assessment of associated risks.

The following list provides an overview on the examples for CRISPR-based tools discussed as relevant in the current literature:

- CRISPR nuclease proteins derived from different source microorganisms: Different ds-nuclease activities of various other naturally occurring CRISPR nucleases, e.g. the SaCas9 variant from *Staphylococcus aureus* which is considerably smaller than the SpCas9 protein, and therefore easier to deliver into the target cells, or the non-Cas9 CRISPR nuclease Cpf1, which generates staggered ds-breaks resulting in “sticky ends” rather than “blunt-ends” ds-breaks as SpCas9 nuclease. Cpf1 is also different from Cas9 as regards other characteristics, e.g. Cpf1 shows an inherently lower incidence to induce “off-target” activity at genomic sequences with non-perfect homology to the intended gRNA recognition sequence (Guha et al. 2017), (Komor et al. 2017).

- Engineered Cas9 proteins with enhanced specificity:
  Engineered variants of Cas 9 were developed like eSpCas9 or spCas9-HighFidelity, with an increased specificity to bind the respective target sequences compared with the unmodified SpCas9 protein (Guha et al. 2017), (Hilscher et al. 2017).
- ss-nucleases developed from mutated variants of Cas9:
  Engineered variants of Cas9 proteins have been developed which have lost one of their two DNA cutting activities and accordingly introduce only single strand breaks into the respective target sequence, i.e. they act as ss-nickases (see (Guha et al. 2017), (Komor et al. 2017). In comparison with ds-nucleases like Cas9 such nickases predominatly trigger HR repair pathways and are therefore characterized by a different ability to generate specific outcomes (Komor et al. 2017).
- Fusions with heterologous nuclease domains:
  A nuclease deficient variant of Cas9 (dCas9) was fused with heterologous nuclease domains, e.g. the FokI nuclease domain, to develop a heterologous site directed paired nickase-system. Such systems can increase the specificity of recognition, since the target DNA is only cut upon dimerization of two components recognizing different adjacent target sequences (see review by (Lee et al. 2016).

In addition to the different CRISPR-systems which are functioning as sequence specific nucleases, the CRISPR-based DNA-targeting system may also be engineered to perform other functions at predefined genomic locations. Sequence-specific DNA binding domains derived from other nuclease systems for genome editing (TALENs, ZFNs, MNs) may in principle be used to create comparable tools to the Cas9-based approaches summarised in the following list. However, CRISPR-based systems are considered the most important, taking into account the breath and speed of technical development and the much higher rate of adoption and use.

- Cas9 variants modified for “base editing”:
  Such approaches were developed by generating fusions of the dCas-protein with heterologous protein domains which promote other genome editing functions, e.g. based on deaminase activity. Deamination of cytidine residues occurring at the targeted genomic locus leads to subsequent base changes at the respective C-positions, in particular cytosine to thymine base substitutions (Komor et al. 2016).
- Nuclease-deficient Cas9-proteins as transcriptional effectors:
  In such approaches a nuclease deficient variant of Cas9 (dCas9) is targeted to specific regulatory sequences to act as an inhibitor of expression upon binding to these regulatory elements (Guha et al. 2017).
- Cas9-based heterologous transcriptional effectors:
  Fusions of dCas9 to transcriptional activators, repressors or protein domains establishing epigenetic modifications at specific target loci to influence gene expression (Guha et al. 2017), (Komor et al. 2017).

An important consideration in addition to the characteristics of the different CRISPR tools is the way of delivery of such tools to the plants cells which are intended to be modified by genome editing.

The most common approach to delivery is the introduction of a recombinant expression construct encoding the necessary components of a CRISPR system (e.g. nuclease, gRNA and donor DNA as appropriate) into the target cells by available GM methods. After selection of stable transformants, regenerated plants are screened for events containing edited sequences at the targeted genomic locations as intended. Since the CRISPR expression cassette is usually not genetically linked to the edited genomic loci the transgenic modification can be removed by segregation during subsequent sexual reproduction steps (HILSCHER et al. 2017).

Alternatively CRISPR components can be introduced transiently e.g. by different viral vector systems also used in VAGE and VIGS applications. Such viral constructs can also be delivered by agroinfiltration methods. Transient expression was shown to be sufficient for initiating genome editing events and might be favoured by developers due to regulatory considerations.

Another route of delivery is the direct delivery of functional ribonuclease complexes consisting of CRISPR-type nuclease protein and the respective gRNA to recipient cells. However, the available methods for direct delivery of such particles involve the use of plant protoplasts and are dependent on the availability of procedures for regenerating plants from protoplast cultures and thus cannot be used in all relevant plant species at present (HILSCHER et al. 2017).

At present a majority of CRISPR-based applications are used to implement SDN-1, SDN-2 or SDN-3 type genome editing approaches. Such applications facilitate different objectives, e.g. to knock out endogenous plant genes by introducing mutations that disrupt the expression of these genes, to insert specific changes to genomic target sequences to modify the gene sequence in a particular way, e.g. to introduce specific new (point) mutations or reversal of mutations present in the recipient genome, or to insert at a certain genomic target locus larger-sized genetic constructs, e.g. transgenic elements, specified by template DNAs provided in addition to the nuclease complex.

With the latter approach all sorts of transgenic constructs can be inserted, including so called gene drive constructs based on CRISPR/Cas9. Such constructs are a recent development designed to invade unmodified genomic target sites during sexual reproduction and thus are passed on in a disproportional high number to offspring individuals as compared to other endogenous genes inherited in a Mendelian fashion (e.g. (CHAMPER et al. 2016, SIMON et al. 2018). By this mechanism gene drive constructs, which may include (trans-)genes for expression of other traits, can spread through (entire) populations quite rapidly.

4.1.2 **Experiences and applications with CRISPR-based systems**

Following the initial description of the system in 2012 (JINEK et al. 2012) CRISPR/Cas based methods for genome editing for application in a wide range of organisms, including plants, were quickly developed.

Recent reviews on the application of CRISPR/Cas based methods in plant research and biotechnology was provided in a study by Hilscher and coworkers (HILSCHER et al. 2017) and a number of other published reviews (MAO et al. 2019, MISHRA&ZHAO 2018, MODRZEJEWSKI et al. 2019, PICKAR-OLIVER&GERSBACH 2019). The simplicity and ease of application of CRISPR-based systems resulted in a widespread and expanding use of the respective

methods in different (crop) plant species. In addition a substantial number of publications are describing new variants of the methodology.

The results of our survey of recent research involving CRISPR applications in plants, which were published between January 2016 and June 2017 are provided in Annex 1. Table 7 lists more than 100 recent research papers relevant to the questions addressed in this study. It is structured firstly according to the different SDN methods which were applied (i.e. SDN-1, SDN-2 and SDN-3 approaches) and the nuclease variants employed and secondly according to the species of plants, which were used in the respective studies.

Plants modified by CRISPR-based genome editing are: apple, *Arabidopsis thaliana*, barley, *Camellia sativa*, cassava, cotton, cucumber, flax (*Linum usitatissimum*), grapefruit, grapevine (*Vitis vinifera*), legumes (*Medicago truncatula*), *Lotus japonicus* (model legume), maize, moss species (*Physcomitrella patens*), oilseed rape, opium poppy, orchid (*Dendrobium officinale*), petunia, poplar (*Populous tomentosa*; chinese white poplar), potato, rice, rubber dandelion, red sage (*Salvia miltiorrhiza)*, soybean, tobacco (*Nicotiana tabacum, Nicotiana benthamiana, Nicotiana attenuata* and wild tobacco), tomato (*Solanum lycopersicum* and *Solanum pimpinellifolium*), watermelon (*Citrullus lanais*) and wheat (bread wheat and durum wheat).

Much of the recent research has been into the improvement of the genome editing system, utilising different sources of Cas9, different nucleases (e.g. Cpf1), nucleases with altered activities (dCas9 and nCas9), fusion proteins with different activities, such as linking dCas9 or nCas9 to e.g. deaminases for the purpose of base editing or fusing Cas9 to marker proteins such as GFP (green fluorescent protein), GUS or proteins conferring herbicide resistance.

Work is also done on optimizing Cas coding sequences for different plants, and to employ different promoters for expression of Cas nucleases, e.g. development specific, controllable, constitutive, or tissue specific promoters. Different systems and vectors are also being developed for purposes of multiplexing, either for editing of large gene families or multiploid crop species. Multiplexed approaches are also discussed for altering multiple components of metabolic pathways. However, such approaches are not yet applied in practice. Multiplexing systems are also being used to induce deletions of large genome fragments, e.g. for knock-out of genes. In contrast to other CRISPR applications for genome editing such approaches are more easily detectable (e.g. see work of YAN et al. 2016 in *A. thaliana*).

Most published research has been for methodology development or basic research. Only very little research is directly linked to the development of products for application in agriculture (see column ‘Purpose of the Study’). An observable trend is that plant offspring which is not containing the transgenic CRISPR constructs is selected early (during first or second subsequent generations) to avoid further mutation events and to create ‘transgene-free’ plants. DNA-free methods, directly applying RNPs (ribonucleoproteins) to protoplast systems were developed in grape, apple, petunia, wheat and maize as another means of ‘transgene-free’ genome editing.

The overwhelming majority of reported applications are SDN-1 knock-outs, often to demonstrate the feasibility of the method in a particular species, or to improve on the method for a particular species (e.g. alfalfa, apple, cassava, cotton, grape etc.). In those cases the genes targeted are intended for reporting purposes only and of no relevance for further

practical application, e.g. in agriculture. Traits that have environmental/ecological relevance include: disease resistance, modulation of flowering onset or flowering time, abiotic stress response (e.g. to cold, drought, frost), stomatal closing, alteration of symbiotic nitrogen fixation, increased shatter resistance of seed (oilseed rape), altered composition of secondary cell wall, male sterility, early maturation and facultative parthenocarpy (UETA et al. 2017 and KLAP et al. 2017 in tomato).

Until May 2018 seven inquiries to APHIS about the regulatory status of different CRISPR applications have been submitted (ECKERSTORFER et al. 2019a), including a mushroom developed with CRISPR/Cas to resist browning WALTZ (2016b), and a CRISPR/Cas waxy corn[1].

4.1.3 Combination of CRISPR-based systems with other approaches

CRISPR-based systems for genome editing are used together with other nGM approaches for a number of different purposes.

A number of other techniques are applied to facilitate the introduction and expression of the CRISPR expression constructs in the targeted plants, plant tissues or cells:

- nGMs used as such are agroinfiltration or approaches like VAGE, i.e. use of viral vectors for gene transfer and expression. Developments of tools for the latter technique are addressed by Lozano-Duran (Lozano-Duran 2016) and Baltes and coworkers (BALTES et al. 2014). Application of such a virus-aided approach for transfer of constructs needed for genome editing is described by BUTLER et al. (2016) for the modification of potato plants for resistance to ALS-based herbicides.
- For most CRISPR-based approaches methods for stable integration of transgenic expression constructs were used, involving among others floral dip methods for *Agrobacterium*-mediated transformation. In addition a number of such techniques is involving the use of protoplast technology and protoplast culture as indicated in Table 7.
- Protoplast technology is used for the DNA-free method of delivery of functional CRISPR-ribonucleoproteins to recipient cells. MALNOY et al. (2016) describe such an approach for genome editing to modify grape vine and apple to induce disease resistance (to powdery mildew and fire blight, respectively).

CRISPR-based systems for genome editing can be applied in combination with oligonucleotides based on the observation that DSBs in the vicinity of the genomic target locus increase the rate of editing events initiated by the oligonucleotides (SAUER et al. 2016b).

CRISPR-based systems for genome editing may also be used for the targeted integration of transgenes at predefined genomic loci in a similar way as ZFN-based techniques (see Chapter 4.3.3). Such CRISPR approaches can facilitate cisgenesis, intragenesis and transgenesis and can also be used for the targeted stacking of genetic constructs at specific genomic loci.

[1] https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/regulated_article_letters_of_inquiry/regulated_article_letters_of_inquiry

Applications of CRISPR technology to knock out endogenous genes can be used as an alternative approach to RNAi based silencing of endogenous plant genes. Such applications can also be used to support approaches like accelerated breeding.

CRISPR-based systems for targeted modification of genomic methylation-patterns can be applied as a tool for facilitating RdDM applications (GUHA et al. 2017).

4.1.4 Detection and identification of CRISPR-based systems applications

Detection and identification of plants developed using CRISPR/Cas systems depend on the type of modifications generated in the genome. SDN-1 and SDN-2 genome editing, although detectable, cannot be distinguished from naturally occurring sequence variations or conventional mutagenesis.

Cisgenes, intragenes or transgenes integrated by SDN-3 genome editing can be detected and identified as any other cisgenic, intragenic or transgenic plant (HILSCHER et al. unpublished).

Depending on the size of deletions and on the availability of information on the specific nature of a deletion introduced by SDN applications, such changes may be detected by methods used for the detection of GMOs.

DNA sequence recognition of CRISPR/Cas systems is dependent on the occurrence of short sequence motives, the so-called protospacer adjacent motives (PAM), in the vicinity of the genomic target sequence (JINEK et al. 2012). If PAM motives can be detected with the appropriate spacing to a putative modified locus, they can provide an additional indication that the modification indeed was introduced by genome editing using a particular CRISPR/Cas system.

4.2 TALE-Nuclease systems for genome editing (TALEN)

4.2.1 Concept of TALE-Nuclease systems for genome editing

TALE-Nucleases are synthetic site directed nucleases developed by coupling DNA-binding domains derived from transcription activator-like effector proteins (TALEs) with a nuclease domain, commonly derived from the FokI endonuclease (see (AGAPITO-TENFEN&WIKMARK 2015). The DNA-binding domain of TALENs is composed of a number of protein modules, which recognize consecutive single nucleotides in a target DNA sequence. The individual DNA-binding domains consist of an array of 15 to 20 different modules and the nucleotide specificity of the coupled modules and the number of modules combined into a DNA-binding domain will specify a particular target sequence of respective length (KANCHISWAMY 2016).

To exert nuclease activity two specifically designed TALEN molecules recognizing neighboring genomic target sequences have to interact. The dimerized FokI domains of both TALEN parts then introduces ds-DNA breaks in the genomic DNA in a spacer region located between both recognition sequences. Due to the combined overall DNA recognition sites of 30-40 nucleotides and the requirement for dimerization TALENs are highly specific for particular genomic locations (GUHA et al. 2017).

Substantial re-engineering efforts are however necessary to tailor TALENs to target different specific recognition sequences, therefore the necessary time and efforts for adaptation are significantly higher than with CRISPR-based systems for genome editing.

Like CRISPR/Cas9 systems TALENs may be used to implement SDN-1, SDN-2 and SDN-3 approaches, depending on the specific application and the provision of specific additional target DNA molecules together with a TALE-nuclease.

Compared with other site directed nucleases TALENs are considered to be less cytotoxic. As other nuclease-based systems they may be associated with off-target activity at genomic sequences with a high, but non-perfect homology to the intended recognition site (KANCHISWAMY et al. 2016). Depending on the nature of the specific target sequence TALENs may show a lower level of off-target activity than other SDNs (KANCHISWAMY et al.).

4.2.2 **Experiences and applications with TALENs**

TALENs have been applied to plant genome editing from 2010 onwards (see (SPRINK et al. 2015), (AGAPITO-TENFEN&WIKMARK 2015). VOGEL (2016) reports that TALEN-based systems have been used in a number of agriculturally used plant species like barley, cabbage, corn, potato, rice, soybean, tobacco, tomato and wheat.

Table 8 lists examples of TALEN applications derived from the literature search as described in Chapter 3.5. Although no publication reports applications for commercial development, the targeted traits could be of agricultural relevance. In addition a number of studies are concerned with basic research, e.g. the identification of TAL effects, the search for TAL effector targets, method development or the functioning of TALEN systems.

Several applications with agricultural relevance were reported: (LI et al. 2012) reported an application to target the recognition site of TAL effectors from *Xanthomonas oryzae* in the promoter of a rice gene which is involved in susceptibility of the crop to the pathogen. Specifically designed TALENs were used to reduce the binding capacity of the pathogen effector proteins. The induced mutations induced heritable resistance to the pathogen which causes bacterial blight.

TALENs were also used successfully in several polyploid crop species with either stable integration or transient introduction of TALEN expression constructs. In wheat resistance to powdery mildew was established by introducing targeted mutations in the three genomic *mlo* homoeoalleles (WANG et al. 2014b), while in potato a herbicide resistance trait was constructed by inducing mutations in the ALS gene (NICOLIA et al. 2015a).

As regards possible attempts to commercialisation of TALEN applications Simplot Plant Sciences as well as Calyxt Inc. issued inquiries to APHIS regarding a potato less susceptible to black spot[2], Iowa State University regarding rice lines and Cellectis plant sciences concerning a soybean product.

[2] https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/regulated_article_letters_of_inquiry/regulated_article_letters_of_inquiry

4.2.3 Combination of TALEN with other approaches

Based on the surveyed literature genome editing by TALEN is combined with other approaches mainly for three different purposes:

Firstly, other techniques are used to introduce and (transiently) express the components of the TALEN system within the targeted plant cells. For this purpose TALEN applications may be combined with techniques directed to transient expression of transgenic TALEN constructs, e.g. by agroinfiltration or virus-aided gene expression (VAGE) according to VOGEL (2012). In a number of other applications methods for stable integration of transgenic TALEN constructs were used, some applications involving protoplast transformation.

Secondly, a number of approaches use TALEN-based techniques to facilitate the generation of transgenic lines (single events or stacked events) with targeted integration of the respective transgenes at predefined genomic loci. According to AGES (2013) such approaches can also be used for the generation of cisgenic or intragenic lines.

Thirdly, TALEN applications may also be applied as a means to generate plant lines with traits which facilitate approaches like accelerated breeding.

4.2.4 Detection and identification of TALEN applications

According to a first estimation by VOGEL (2012), genetic modifications produced with TALEN in plants can be detected, if information on the specific genetic modification is available. As with other genome editing approaches the possibility for identification depends mainly upon the extent of the genetic modification at a specific site and the occurrence of recombinant sequences at the modified sites.

4.3 ZincFinger Nuclease (ZFN) systems for genome editing

4.3.1 Concept of ZFN systems for genome editing

Zinc finger nucleases (ZFNs) are synthetic nucleases, which consist of two independent subunits and can introduce ds-breaks into target DNA after binding at a specific genomic locus as protein dimers. Similar as described for TALENs each subunit is composed of a DNA-binding domain and a nuclease domain (ECKERSTORFER et al. 2014).

The DNA-binding domains are derived from a specific class of eukaryotic transcription factors, so called zinc finger (ZF) proteins. The utilized ZF domains are quite small (composed of approximately 30 amino acids) and have the ability to bind to stretches of three specific nucleotides (nt), which are occurring in the respective sequence elements targeted by regulatory ZF-proteins. ZF domains were selected because they can be linked together in deliberately assembled arrays to recognize novel target DNA sequences (CURTIN et al. 2012).

Insight into the sequence recognition capacity of different ZF domains enabled the assembly of recombinant DNA-binding elements composed of several specific ZFs with appropriate target sequences (AGAPITO-TENFEN&WIKMARK 2015). ZFNs usually contain arrays of 3-6 ZFs linked together, the resulting ZF-domains are therefore targeting DNA recognition sequences with a length of 9-18 nt.

The nuclease domain is usually derived from the endonuclease FokI and does not have any own DNA specificity. To be able to cut DNA a dimer of two nuclease domains must be bound to the target DNA sequence. Therefore a functional ZFN is composed of a heterodimer of two separately designed ZFN parts recognizing 9-18 nt of a specific DNA sequence separated by a short 5-7 nt spacer sequence, which is occupied by the FokI dimer.

Specificity of the ZFN complexes can be achieved by using two variants which would only be active when assembled as heterodimer at their target site, or by using homodimeric nuclease variants, which will only dimerise after being directed to their target sites by the specifically designed ZF-domains (GUHA et al. 2017). It was however noted that due to the intrinsic properties of the ZF-domains to mostly target guanine-rich sequences the range of potential recognition sites is more limited as with CRISPR-based systems or TALENs (KANCHISWAMY 2016). In addition the custom design of ZFNs with new sequence targeting specificities is quite laborious and complex and a substantial number of specifically designed ZFNs may not be functioning well enough for a desired application (CURTIN et al. 2012).

In addition ZFNs may result in significant off-target activity and therefore higher levels of cytotoxicity (PODEVIN et al. 2013).

ZFNs can be used to implement SDN-1, SDN-2 and SDN-3 approaches like other SDN systems, depending on the specific application and the provision of specific additional target DNA molecules together with the ZFN nuclease.

4.3.2 **Experiences and applications with ZFNs**

The possibility for application of SDN approaches based on ZFNs in plant species was demonstrated around 2005 in initial studies in Arabidopsis and tobacco (see Vogel 2016). Since then ZFNs have been applied for GE in several crop plant species, like maize and soybean, targeting relevant traits like herbicide tolerance and modified composition which might be commercially introduced (AGAPITO-TENFEN&WIKMARK 2015).

Table 9 lists specific examples of ZFN applications derived from the literature search.

In the retrieved publications ZFN approaches are used in model plants (Arabidopsis and tobacco) as well as in crop plants (soybean, maize, tomato, rice) and in trees, including fruit trees (apple) and poplar trees.

The research was conducted for a number of different purposes:

- Several reports are dedicated to method development, e.g. to increase efficiency of genome editing, to develop specific approaches for research (among others functional genomics) and to develop approaches for multiplexed genome editing.
- Some papers address issues of basic research: e.g. to investigate and understand gene families, DNA repair pathways and silencing mechanisms or major transcription factors and the pathways linked to them, especially in polyploid genomes like the soybean genome.
- Few projects describe the application of the technique for the development of traits: mostly SDN-1 applications to knock out particular genes and to introduce mutations into genes of interest (directed mutations were achieved via SDN-2), e.g. herbicide resistance.

- Several publications address the development of methods for molecular stacking of genetically linked transgenes at specific genomic locations (in particular in maize and rice).
- The latter publications document the relevant interest from commercial institutions, in the development of genome editing methods for targeted integration of transgenes (SDN-3 applications). However, the surveyed published literature describes only proof of principle experiments for the development of methodologies and so called 'trait landing pads' (TLPs), designed for the molecular stacking for multiple transgenes. The retrieved literature did not yet cover the use of this approach for the development of plants with traits relevant for use in agriculture.

For purposes of commercial application a variety of HT maize were developed with ZFN-based EXZACT™ Precision Technology[3]. According to an inquiry to APHIS the approach was also used to develop a maize line with reduced phytate content (CAMACHO et al. (2014).

4.3.3 Combination of ZFN with other approaches

Based on available literature ZFN was combined with other approaches mainly for three different purposes:

Firstly, other techniques are used to introduce and (transiently) express the components of the ZFN system within the targeted plant cells. For this purpose ZFN applications may be combined with techniques directed to transient expression of transgenic ZFN constructs e.g. by agroinfiltration (see Table 9) or virus-aided gene expression (VAGE) according to VOGEL (2012). In a number of other applications methods for stable integration of transgenic ZFN constructs were used, some applications involving protoplast transformation (see Table 9).

Secondly, a number of approaches use ZFN-based techniques to facilitate the generation of transgenic lines (single events or stacked events) with targeted integration of the respective transgenes at predefined genomic loci (see Table 4). According to AGES (2013) such approaches can also be used for the generation of cisgenic or intragenic lines.

Thirdly, ZFN applications may also be applied as a means to generate plant lines with traits which facilitate approaches like accelerated breeding (see Table 9).

4.3.4 Detection and identification of ZFN applications

Detection and identification issues for ZFN applications are similar as for other discussed SDN applications.

4.4 Meganuclease-based systems for genome editing

4.4.1 Concept of Meganuclease systems for genome editing

Meganuclease is a commonly used term for endonucleases, which are highly specific for target recognition sequences which are 12-40 nt long and therefore substantially longer than sequences targeted by other bacterial endonucleases (VOYTAS 2013). Some of these

[3] www.exzactprecisiontechnology.com

meganucleases, like the yeast derived I-Cre1 meganuclease are encoded by “selfish” genetic elements, e.g. intron sequences which can reinsert themselves into copies of the genes, which have lost the respective introns earlier due to action of the meganuclease encoded within the intron. I-Cre1 is initiating the integration of the intron sequence by introducing ds-breaks into the ribosomal RNA gene harbouring the recognition sequence and thereby promoting integration of the intron sequence through HR. Such meganucleases are naturally occurring mediators of gene targeting and in reference to this function the name “homing endonuclease” is frequently used in the literature (e.g. (VOYTAS 2013).

A significant number of different meganucleases have been identified, targeting different sequence motives (AGAPITO-TENFEN&WIKMARK 2015), however due to the size of their recognition sequences the respective target sequences occur only rarely in genomic DNA.

Meganucleases are typically acting as homodimers combining two identical subunits. They are smaller in size than other genome editing reagents (VOYTAS 2013) and thus e.g. have advantages concerning delivery to target cells as functional nucleases. However, they do not have a modular structure and the recognition and the DNA cutting domains are not separated. Therefore, re-engineering to develop functional variants with different recognition sequences is challenging (GUHA et al. 2017). Reviews note efforts to develop meganucleases with changed specificities, particularly using I-Cre1 and related homologs as source material. However, only few reports exist towards application of such re-engineered meganucleases in genome editing (AGAPITO-TENFEN&WIKMARK 2015),

4.4.2 **Experiences and applications generated with Meganucleases**

According to DABOUSSI et al. (2015) meganuclease methods have been used as early as 1993 to enhance the frequency of homologous recombination events at specific genomic loci, e.g. in tobacco plants.

Since DSBs by meganucleases led to a higher frequency of homology directed repair events than other site-directed nuclease systems further research was directed to the application of meganuclease systems for the targeted integration of transgenes at selected genomic locations and to targeted stacking of different transgenes, like by inserting two transgenes resulting in expression of herbicide resistance adjacent to a pre-integrated transgenic trait for insect-resistance in cotton (DABOUSSI et al. 2015). As referred to above in Chapter 4.3.2 the use of meganucleases has been explored for this purpose as an alternative to e.g. ZFN-based approaches (NANDY et al. 2015).

A review of scientific literature on the application of MN-based systems for genome editing in plants retrieved only a limited number of additional relevant publications. Based on the applications listed in Table 10 MN-based approaches were used for basic research in model plants as well as for research in relevant crops, e.g. maize and barley, which can provide a basis for further product development, e.g. the development of maize lines with male sterility.

In addition the literature review retrieved a number of publications which focused on relevant aspects for the further development of MN-based genome editing methods, among them the establishment of a database containing information on different meganucleases and their properties (TAYLOR et al. 2012), and their use to facilitate the assembly of transgenic constructs from sequence elements encoding standardized building blocks for functional domains and vectors to transfer such constructs (LI et al. 2014, LIU et al. 2014).

Meganucleases were also used to design transgenic constructs for gene drive applications in mosquitos (WINDBICHLER et al. 2011).

4.4.3 Combination of meganuclease approaches with other approaches

Meganuclease techniques can be used to achieve site-specific integration in combination with cisgenesis and intragenesis. Virus-aided gene expression may be used to transiently express meganuclease constructs in recipient cells to initiate genome editing events (VOGEL 2012).

4.4.4 Detection and identification of MN applications

Detection and identification issues for MN applications are similar as for other discussed SDN applications.

4.5 Oligonucleotide Directed Mutagenesis (ODM)

4.5.1 Concept of ODM

ODM is a method to introduce targeted mutations into specific genomic sequences of microorganisms, animal and plant cells using a similar general approach: oligonucleotides containing a modified nucleotide sequence are introduced into cells and are instructing *in vivo* genome editing processes at the sites of close sequence homology to the used oligonucleotides. Such processes lead to insertions of one or few additional nucleotides or the deletion of one or few nucleotides depending on the sequence of the used oligonucleotides, i.e. the sequence alterations *vis a vis* the targeted genomic sequences. Such changes can lead to the expression of gene products with a modified sequence or to the repair of previously mutated genomic sequences (e.g. (LUSSER et al. 2011b), (BREYER et al. 2009), (RIVERA-TORRES&KMIEC 2016). Silencing of endogenous genes can be achieved by introducing stop codons, frameshift mutations or deletions interrupting the reading frame of the target gene (KMIEC 2003).

In the literature a variety of different names are listed, which are used in different sources to describe this technique (LUSSER et al. 2011a) (HEINEMANN 2015).[4]

4.5.2 Experiences and applications with ODM

The concept of ODM dates back to the late 1970s (PAUWELS et al. 2014), and the term ODM was coined for *in vivo* applications of the technique around 1986 (Riviera-Torres & Kmiec 2015). Around 1990 *in vivo* homology-dependent, recombination-mediated methods were routinely used in the yeast *Saccharomyces cerevisiae* to generate genomic modifications, including insertions and deletions (HEINEMANN 2015).

[4] Among others the following names are used to describe oligonucleotide based mutation approaches: oligonucleotide-mediated gene modification, targeted gene correction, targeted gene repair, RNA-mediated DNA modification, RNA-templated DNA repair, induced targeted mutagenesis, targeted nucleotide exchange, chimeraplasty, genoplasty, oligonucleotide mediated gene editing, chimeric oligonucleotide dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotides induced recombination, oligodeoxynucleotide-directed gene modification, therapeutic nucleic acid repair (LUSSER et al. 2011b)

Practical protocols for application of the ODM technique to a broader range of organisms appeared more recently (PAUWELS et al. 2014). There is some experience with ODM in model plant systems, like Arabidopsis and tobacco, as well as in crop plants. However, published data on the use of ODM in plants is more limited than in bacteria, yeast, and mammals (SAUER et al. 2016a).

A literature search did not reveal many applications in addition to the ones already addressed in prior reviews (AGAPITO-TENFEN&WIKMARK 2015, LUSSER et al. 2011a, VOGEL 2016) and those listed in Table 11 as commercial developments.

The development of the RTDS™ (Rapid trait development system)[5] method has significantly advanced the effectiveness of the ODM approach for the modification of crop species (GOCAL et al. 2015). RTDS™ was developed for commercial use by the US based company Cibus which also operates a branch in the Netherlands. RTDS™ was used to e.g. create an oilseed rape line, SU Canola™, which was modified for resistance to sulfonylurea herbicides. SU Canola™ is already marketed in the US[6] and has received authorisation for commercial use in Canada (SCHAART et al. 2015).

In addition three other RTDS™ products are expected to be introduced to the market in the next years: glyphosate tolerant flax, herbicide tolerant rice and phytophthora-resistant potato. The developer has conducted field trials in Sweden and the UK (ABBOT 2015).

4.5.3 Combination of ODM with other approaches

As pointed out by SAUER et al. (2016b) and RIVIERA-TORRES & KMIEC (2015) the introduction of DSBs near the target site, i.e. the site of intended change, will increase ODM's efficiency, which is typically quite low. The approach to simultaneously applyODM together with SDN techniques will probably increase efficiency of genome editing applications.

4.5.4 Detection and identification of ODM applications

ODM plants can be detected if information on the nucleotides in the vicinity of the mutation is available. However, identification of ODM plants is not possible since they are, at the molecular level, not distinguishable from plants developed by conventional mutation breeding. The same holds true for spontaneous mutations or single nucleotide polymorphism mutations (LUSSER et al. 2011a, VOGEL 2012).

4.6 Multiplex Automated Genomic Engineering (MAGE)

4.6.1 Concept of MAGE

While the ODM technique described in the previous chapter mainly focuses on introducing specific changes at single genomic loci, targeted genome-scale engineering methods based on the use of sequence-modified oligonucleotides have been established in non-plant systems (e.g. bacteria) to simultaneously change multiple independent genome loci (ESVELT&WANG 2013). Multiplex Automated Genomic Engineering (MAGE) is an example of

[5] https://www.cibus.com/index.php

[6] https://www.falcoseed.com/canola/

a cyclic process for such a method enabling efficient multiplex genome editing. It is based on using pools of single-stranded oligonucleotides with overall sequence homology to target several different genes in bacterial genomes in parallel (WANG et al. 2009). These oligonucleotides contain in their central region different sequence aberrations which lead to accumulation of combinations of mutations in different cells undergoing the process. In bacterial systems this process can be automated to facilitate rapid and continuous generation of a diverse set of genetic changes (e.g. mismatches, insertions, deletions) in a defined number of target genes (WANG et al. 2009).

The method was developed to rapidly modify different components of complex biosynthetic pathways in bacteria in parallel (Wang et al. 2009). However oligonucleotide-mediated genome engineering approaches such as MAGE are mechanistically simple and may be applicable in all systems where oligonucleotide-mediated allelic replacement has been demonstrated (ESVELT&WANG 2013).

4.6.2 **Experiences and applications with MAGE**

The method was first used to engineer a biosynthesis pathway in *Escherichia coli* to overproduce the isoprenoid lycopene, an industrially important substance (WANG et al. 2009). In a very rapid process, which generated a very high number of combinatorial genomic variants in 24 pathway components, bacterial cells could be isolated within 3 days, which showed a more than fivefold increase in lycopene production. This can be seen as a significant improvement over existing one-step metabolic engineering techniques (ESVELT&WANG 2013).

To our knowledge MAGE was not adapted or applied to modifiy plants yet. However, MAGE might represent an example for future approaches for multiplexed genomic engineering, which are simultaneously targeting different genetic components in plant cells and thus introducing substantial change in phenotypic performance in a short time.

4.6.3 **Detection and identification of MAGE applications**

Since we did not find literature reporting MAGE applications in plants, no discussion of issues concerning detection is available. However, highly multiplexed approaches to GE like MAGE will create a number of specific genetic changes at different genomic loci, which may be individually detected by existing methods upon knowledge on the relevant sequence modifications. Together the multiple changes could be used as a fingerprint pattern to aid the identification of organisms developed by MAGE-like approaches. The probability that identification can be achieved is dependent on the number of individual sequence changes and their genomic location. If a higher number of specific intended changes is present in a modified plant (usually 3 or more), the identification of such applications is possible with a sufficient degree of certainty.

4.7 RNA dependent DNA Methylation (RdDM)

4.7.1 **Concept of RNA dependent DNA Methylation (RdDM)**

RdDM applications operate via an endogenous system of plants to control the expression of specific genes by epigenetic mechanisms, particularly by RNA-induced transcriptional gene

silencing. This system prevents expression of foreign DNA, e.g. viral DNA, and controls the activity of repetitive DNA and transposable elements, which may otherwise negatively affect genome stability (LAW&JACOBSEN 2010). Epigenetic control is affected by targeted methylation of specific DNA sequences, e.g. in promoter regions, inhibiting the expression of the respective genes without introducing changes of the DNA sequence itself. Small noncoding RNAs are involved in initiating a complex process to establish sequence-specific DNA methylation patterns by directing DNA methylating enzymes to specific genome loci (ZHANG&ZHU 2011), (MATZKE&MOSHER 2014).

To artificially induce this system dsRNAs need to be expressed, which are homologous to the promoter regions of the targeted genes. Typically expression constructs for such dsRNAs or artificial microRNAs are introduced into the recipient plants as stably inserted transgenes or as part of transiently present extrachromosomal elements. This requires that at some point the recipient plant is genetically modified (AGES 2012).

The expressed RNAs are then processed by the DICER enzyme complex, an element of the RNAi mechanism in plants, giving rise to small fragments (small interfering or siRNAs) of about 24 nt in size. The siRNAs associate with cellular components of the RNA-induced silencing complex (RISC) and direct the generation of epigenetic modifications at complementary regulatory DNA sequences (ZHANG&ZHU 2011). These *de novo* DNA methylation and histone modification signals then interfere with the expression machinery thus silencing the expression of the respective genes.

In plants specific methylation patterns can be passed on to offspring generations; induced changes in DNA methylation were found to be transmissible for at least a number of generations (HAUSER et al. 2011), (MATZKE&MOSHER 2014). Thus traits generated by a specific RdDM application are retained for a number of generations, even when the initiating transgenic modification is lost by segregation during sexual reproduction (SCHAART et al. 2015).

According to VOGEL (2016) propagation of RdDM-mediated traits in the absence of the trigger were demonstrated in petunia, rice and tomato. However, the underlying mechanisms are not understood well enough to predict the stability of RdDM-generated traits and the influence of environmental conditions on the expression of such traits with sufficient certainty.

4.7.2 **Experiences and applications generated with RdDM**

The concept of RdDM was developed some 15 years ago and since then several RdDM applications have been studied in the following plant species according to AGES (2013) and (VOGEL 2016): Arabidopsis, carrot, petunia, maize, barley, wheat, potato, rice and tomato.

Table 12 below lists examples of research on RdDM derived from the literature.

For purposes of this study RdDM is referred to as a method to induce site-specific methylation and thus silencing of the targeted promoter(s) by transcriptional gene silencing (TGS).

Three main methods for initiation of TGS events are described in the retrieved literature:

- VITGS (virus-induced TGS), an approach which facilitates that the following generation (S1) will not contain an siRNA donor;

- Transgrafting, where even the first silenced generation (S0) will not contain an siRNA donor construct, though siRNA will be present;
- Transgene-mediated transcriptional gene silencing based on a silencing construct stably integrated into the plant genome.

The latter approach is mostly used for experimental purposes and not intended to study heritability of gene silencing nor obtaining heritable gene silencing without a transgenic modification present.

Transgene-mediated TGS is being used by DuPont Pioneer for the purpose of hybrid seed production, using reversible male sterility as a genetic method to replace detasseling of maize for seed production (CIGAN et al. 2014).

Cases of RdDM-mediated gene silencing where the effect is “inherited”, i.e. present also in the next generation(s) has mostly been shown for silencing of the expression of transgenes, rather than for endogenous plant genes. Transgenes are thought to be more susceptible to silencing than endogenous genes. Gene silencing effects in plants of the next generation after initiation of RdDM by VITGS were found only for a few endogenous genes: for chalcone synthase (CHS-A) in petunia and for colorless non-ripening (LeSPL-CNR) in tobacco.

Most of the work reported in the retrieved publications has been carried out to improve the VITGS method and to investigate why endogenous genes are much more resistant to hereditary TGS, investigation among others methylation patterns, effects of siRNA size and position within a promoter, histone modification, or cellular components of different methylation pathways.

According to SCHAART (2015) RdDM has not been applied for commercial purposes yet.

4.7.3 **Combination of RdDM with other approaches**

The RdDM approach relies on the expression of a dsRNA precursor gene which may be achieved by modification of the respective plant by cisgenesis, intragenesis or virus-aided gene expression (VOGEL 2012)

4.7.4 **Detection and identification of RdDM applications**

Since the RdDM approach does not alter the DNA sequence, applications cannot be detected by standard methods as for the detection and identification of GMOs or nGM applications, which contain characteristic DNA sequences generated by the method that can function as a specific “fingerprint” to distinguish them from any other conventionally established line of the same crop species.

Methods which can detect different DNA methylation patterns in modified and non-modified plant lines were developed for scientific research; however, the respective methods are not yet adapted for routine use.

4.8 Other techniques for modification of gene expression

4.8.1 Virus-Aided Gene Expression (VAGE)

VAGE involves the use of vectors developed from plant viruses in order to transiently introducte and express foreing genes in somatic plant cells. While the stable integration of such vectors is not foreseen (VOGEL 2016), it cannot be excluded to happen with a low frequency. The method can be adapted silence specific endogenous plant genes which is referred to as virus-induced gene silencing or VIGS (VOGEL 2016).

Different viral vector systems developed from both RNA and DNA plant viruses may be used, depending on the crop plant of interest. A review of available systems and a discussion of the limitations as regards the availability of appropriate vectors are provided by SENTHIL-KUMAR & MYSORE (2011). Vectors of choice should not elicit relevant disease symptoms in the treated plants, they can be used in different plant species (VOGEL 2016).

Further considerations as regards VAGE are the duration of the infection caused by the vector, the possibility to spread throughout the plant tissues, the ability of the vector to be transmitted to offspring plants via seed and the capacity of the vector system to transfer additional DNA, i.e. the respective gene expression construct. As noted for SDN-based systems for genome editing, VAGE vector systems can transfer the small sized meganuclease expression units, but some of them have a limited capacity for the larger sized CRISPR/Cas and TALEN reagents (VOYTAS 2013).

Delivery of the viral vectors can be achieved with different methods, including inoculation methods, biolistic transfer and agroinfection using T-DNA constructs, which harbour the viral vector sequences (VOGEL 2016).

4.8.2 RNAi-based gene silencing

Plants contain a natural RNAi-based mechanism for post transcriptional gene silencing (PTGS). It functions e.g. to prevent the expression of viral genes to make plants resistant to virus infections and to differentially express endogenous genes in certain tissues and at certain developmental stages. An overview of the specifics of the mechanism is presented in (HILSCHER et al. 2017).

The RNAi machinery of plants can be used to silence specific genes of interest. The mechanism is triggered by small dsRNAs, which contain sequences homologous to parts of the coding regions of their target genes. Such dsRNAs can be microRNAs involved in plant development or stress response pathways or small interfering siRNAs derived from viral RNA, naturally occurring antisense transcripts, or transposon sequences. In a number of steps and involving a complex cellular machinery these RNAs are processed and finally loaded onto a silencing complex, which targets and destroys specific mRNAs or inhibits their translation.

The first step involves DICER type endonucleases, which recognise the dsRNAs and process them into small fragments with a length of 21-25 nt. Subsequently the trimmed RNAs associate with the other components of the so called RNA induced silencing complex (RISC), including members of the Argonaute family of proteins, which possess domains exhibiting RNase activity. The RISC complex mediates target sequence recognition and according to the function of the specific complex can initiate PTGS or transcriptional gene

silencing (TGS). For TGS genomic DNA sequences are targeted and modified with epigenetic signals, like DNA methylation (see Chapter 4.7.1). For PTGS mRNAs are targeted and cut or destabilised.

In effect all triggered pathways act to silence the expression of specific genes. In plants RNA-dependent polymerases can amplify the silencing signals and the small RNAs may be transported through the plant and induce systemic effects.

As outlined in HILSCHER et al. (2017) the above described mechanism can be triggered with synthetic dsRNA constructs to target different genes of interest:

These genes may be endogenous plant genes and the silencing of such genes may generate phenotypic traits like different composition, abiotic stress tolerance or disease resistance, e.g. to fungal or bacterial pathogens.

RNAi applications may also target RNAs from plant pathogens, e.g. viruses, and the destruction of viral RNAs can lead to virus resistant plants.

A third type of applications is targeting plant pests, like insects, fungi or nematodes. Ingestion of plant material containing siRNAs can lead to silencing of genes in the exposed pest individuals, which are vital for their survival, reproduction or can influence their behaviour.

4.8.3 CRISPR-based modification of gene expression

As indicated in Chapter 4.1.1 CRISPR-based modification of gene expression can be achieved by coupling a sequence specific CRISPR reagent with disabled nuclease domains, i.e. a dead Cas protein to effectors which act as transcriptional activators, transcriptional repressors or effectors (e.g. methylase-, demethylase-domains). These effectors introduce site-specific epigenetic modifications to influence the expression of the targeted endogenous plant genes (for overview see KOMOR et al. 2017).

4.8.4 Experiences and applications generated by VAGE, RNAi-based gene silencing and CRISPR-based modification of gene expression

VAGE-techniques have been used for approximately 20 years with a variety of vectors being employed for different applications (VOGEL 2016). VAGE can be regarded a support technique for other nGM applications, among them SDN applications, RNAi-based techniques, RdDM, and induction of early flowering for accelerated breeding purposes.

RNAi-based gene silencing was used quite early in plant breeding, with first applications (FlavrSavr™ tomato) introduced for marketing in 1994. Examples for the use of targeting (i) the modification of compositional content in potato, rapeseed, tomato, wheat, rice, apple, and carrot; (ii) modification to induce draught tolerance in rapeseed, maize and potato; (iii) resistance against plant viruses in barley, cassava, tomato and wheat; (iv) resistance against fungal pathogens in barley and wheat; (v) resistance against bacterial pathogens in rice; resistance against pest insects in maize and wheat and (vi) resistance against nematodes in soy (HILSCHER et al. 2017).

The review by HILSCHER et al. (2017) also lists RNAi-based transgenic crops which were approved for commercial purposes or agronomic evaluation. Those are plum resistant to plum pox virus (USA), common bean resistant to bean golden mosaic virus (Brazil), maize

resistant to western corn rootworm (USA) which is also notified for import into the EU, Innate™ potato with impaired black spot bruise development (USA), Arctic™ apple with impaired enzymatic browning of apple flesh (USA, Canada), alfalfa with reduced lignin content (USA), soybean with altered fatty acid content (EU, USA) and FlavrSavr™ tomato with decreased cell wall breakdown (USA).

CRISPR-based techniques for modification of gene expression are currently being developed at a fast pace (e.g. KOMOR et al. 2017), however the technique was only recently conceived as a research tool and is still in a proof of concept stage. Widespread use of CRISPR-based techniques and the ease and versatility of the system to adapt to different applications will probably be drivers for further development and application.

4.8.5 Combination of VAGE, RNAi-based gene silencing, and CRISPR-based modification of gene expression with other approaches

VAGE/VIGS can be used in combination with SDN applications, RNAi-based techniques, RdDM, and induction of early flowering.

According to VOGEL (2012) VIGS and RNAi-based gene silencing can be combined with reverse breeding, RdDM and accelerated breeding.

CRISPR systems for site directed methylation or demethylation can be used to facilitate RdDM (GUHA et al. 2017).

4.8.6 Detection and identification of applications

VAGE constructs can only be detected and identified in plant cells which still contain viral vector DNA after the modification step.

For the identification of RNAi plants event-specific detection methods can be applied based on the information on the transgene and the integration site.

CRISPR-based constructs for control of gene expression can only be detected and identified if the respective CRISPR expression construct is stably inserted in the plant genome.

4.9 Cisgenesis/Intragenesis

4.9.1 Concept of Cisgenesis/Intragenesis

Cisgenic and intragenic crop plants are generated by a similar methodological approach as transgenic plants. However, in cisgenesis and intragenesis only genetic material derived from the gene pool of the recipient species and/or of sexually compatible species, i.e. closely related cultivars or wild species, are used as a source for the recombinant constructs to be inserted. Cross-species or cross-kingdom gene transfer, which is common in transgenic approaches, is not undertaken.

In cisgenesis only copies of naturally occurring genes are used for transformation, including their native non-coding and regulatory elements, i.e. promotor and terminator sequences, in their original orientation. The source of genes used for gene transfer is therefore limited to the range, which is also available for conventional plant breeding approaches. However, in

cisgenesis single genes are isolated from the genome and transformed back into the recipient plant without further altering the genetic background of an established plant variety.

For the construction of intragenic plants the recombination of different genetic elements derived from all genes of the recipient genome is possible. Therefore naturally occurring functional elements originating from all individual genes of the recipient species or sexually compatible species may be combined. This is increasing the possibilities to establish recombinant constructs, which e.g. do not contain non-coding sequences or which are regulated in a different way compared with the native genes, composed of different naturally unlinked genetic elements or contain native genetic elements in reverse orientation.

The advantage of intragenesis/cisgenesis is that it avoids potential 'linkage drag' associated with classical cross-breeding. This unwanted introduction of alleles from non-elite crossing partners usually has to be removed via multiple steps of backcrossing. Depending on the propagation system of the crop this approach can be used as a tool to transfer genes between related plants faster than with traditional breeding approaches in addition to being considered ethically more acceptable. Thus it is particularly attractive for crops which are mostly propagated vegetatively (e.g. potatoes, bananas), crops with a long generation time (e.g. trees, grapevines) or crops with complex genetics, like a high polyploidy status, (e.g. barley) (ECKERSTORFER et al. 2014). Consequently cisgenesis/intragenesis is tested in particular in fruit trees, but also forest trees (e.g. poplars) are being improved by these approaches (AGES 2012). While cisgenic approaches are particularly attractive for introducing resistance genes into elite susceptible crop cultivars, intragenic approaches are mainly used for gene silencing.

4.9.2 **Experience and applications generated with Cisgenesis/Intragenesis**

The concept of intragenesis was first introduced as 'all-native DNA transformation' or 'intragenic modification' by Rommens (ROMMENS 2004, ROMMENS 2007) and the concept of cisgenesis became internationally known through the publications of SCHOUTEN et al. (2006 a&b). Although the concepts of cisgenesis and intragenesis demand more proficiency in the development of new cultivars compared to transgenic plants, today some applications achieved with this kind of techniques are quite advanced. Some cisgenic and intragenic plants have already been released for experimental purposes in the EU and even been authorized for cultivation in the US (see also Holme et al. 2013, (VOGEL 2016).

The focus of plant breeders and researchers working on the development of cis- and intragenic crops currently lies on the improvement of disease (viral and fungal) resistance and quality traits (e.g. improved fodder characteristics). While usually the modifications aim at the overexpression of existing genes or the expression of a new gene from the plant breeder's gene pool (e.g. resistance traits) sometimes also gene silencing approaches are chosen (e.g. reduced lignin content in alfalfa).

As a prerequisite for the application of cis-and intragenic approaches the respective genes of interest need to be identified and sufficiently characterized. Currently a lot of effort in plant breeding is focused on the sequencing and assemblage of the structure of various relevant plant genomes and on specifying the function of the identified genes. Thus an increasing number of isolated and well characterized genes are becoming available for crop

improvement with cis- and intragenesis. However, the lack of knowledge on regulatory sequences still hampers their application in many crop plants (Espinoza et al. 2013).

VOGEL (2016) and SCHAART et al. (2016) report field trials with cisgenic scap-resistant apple and cisgenic apple with increased anthocyanin content (Netherlands), cisgenic fire blight resistant apple (Switzerland), cisgenic phytophthora resistant potato (Belgium, Netherlands, Switzerland) and cisgenic barley with improved phytase activity (Denmark). Some cisgenic plants have reached an advanced phase of development (e.g. scab resistant apple, late blight resistant potato) and consequently the focus is on their further improvement (i.e. removal of selective markers, detailed molecular characterization) and their functioning/applicability (e.g. durable resistance by gene pyramiding) (see Annex 7, table 13). Research on the late blight resistant potato addresses e.g. risk management options by evaluating the application of various cisgenic cultivars differing in the kind and number of introduced R-genes in cultivation rotation schemes adapted to prevalent pathotypes (SCHAART et al. 2016). In other plant species preparatory work for a potential cis- or intragenic modification are being pursued: e.g. research to identify candidate genes and regulatory sequences (e.g. promotors) as well as the development of intragenic vector systems.

Tables 13 and 14 provide an overview on the results of a literature survey of applications of cisgenesis and intragenesis, respectively.

Worldwide a number of plant species are being improved applying cis- or intragenesis, e.g. strawberry, melon, citrus plants, poplar, alfalfa, pasture grass and perennial ryegrass (ECKERSTORFER et al. 2014) (VOGEL 2016). In addition cis- and intragenic approaches are developed for sugar cane, maize, Chinese cabbage and banana. In the US an intragenic potato resistant to the potato virus Y is being developed (CAVATORTA et al. 2011, DUAN et al. 2012).

The Simplot company[7] developed the Innate potatoes (intragenic with reduced acrylamide levels), available on the market in the USA. The first generation contains lower levels of asparagine and is less susceptible to black spot, the second generation also exhibits resistance to phytophthora (VOGEL 2016).

In the UK a field trial has been authorised to be conducted from 2017 until 2021 to test a potato line that is blight and nematode resistant, less susceptible to black spot, and produces less acrylamide when cooked at high temperatures (CROP BIOTECH UPDATE 2017).

CAMACHO et al. (2014) report on two inquiries to APHIS as regards their status of regulation: cisgenic scab resistant apple developed at Wageningen University in the Netherlands and intragenic grapes with increased anthocyanin production developed by the University of Florida.

4.9.3 **Combination of Cisgenesis/Intragenesis with other approaches**

AGES (2012) states that although other new techniques could in principle be combined with cisgenesis, this is unlikely at present because of technical constraints. Possible applications

[7] http://www.simplot.com/plant_sciences/

listed are the combination of cisgenesis and ODM or the combination of cisgenesis and agroinfiltration.

According to AGES (2013) intragenesis may be used as a technique to support applications aimed at silencing endogenous genes, e.g. for reverse breeding.

4.9.4 **Detection and identification of Cisgenesis/Intragenesis applications**

Detection and identification are possible with PCR methods if gene sequence and place of insertion of the cisgene or intragene respectively are known (LUSSER et al. 2011a, VOGEL 2012). According to (AGES 2012) information on the cisgene and the flanking regions of the insertion site need to be available for the development of event-specific methods for detection and identification.

4.10 Transgrafting

4.10.1 **Concept of Transgrafting**

Transgrafting describes the combination of grafting, which is a traditional horticultural technique practiced in particular with fruit trees and ornamental plants, such as roses, with genetic modification of plants parts used in the grafting process. During this process a bud-bearing plant part, the scion, is placed onto a rootstock derived from another related plant individual. This way the vascular tissues of both plant parts come into contact and if vascular connection is established between them a chimeric plant is produced.

For transgrafting one or both of these parts is/are genetically modified for the expression of specific traits in the respective plant parts. However, in most applications of the technique a non-modified scion is grafted onto a GM rootstock (SCHAART & VISSER 2009). Grafting and thus transgrafting can only be used in dicotyledonous crop plants.

Transgrafting is conducted to pursue a number of different objectives in plant breeding and production:

The GM rootstocks may be modified to induce traits that increase the fitness of the rootstock and thus the overall plant in the growing environment, without introducing genetic modifications in the fruit-bearing parts of the plant or the harvested (fruit) products. The used rootstocks are genetically modified for resistance to environmental stress, soil-borne diseases or may display improved rooting ability (see VOGEL 2016). In this way scions selected for their stem, leave, bud or fruit characteristics may be combined with rootstocks modified for pest or disease resistance or rooting characteristics.

GM rootstocks may also be used to indirectly improve the performance of non-GM plant parts, without the need to genetically modify the scions themselves (LUSSER et al. 2011b). To achieve this the used GM rootstock is modified to express transgenic proteins or RNAs, or produce specific compounds due to the genetic modification (e.g. plant hormones), which are transported into the non-GM parts of the plant and improve specific traits of the scion. A pathway to modifying gene expression in the scion is the production of regulatory RNAs in the GM rootstock, which are influencing, e.g. the epigenetic regulation of gene expression in scion cells after transport into the non-GM modified parts of the plant (BAI et al. 2011).

Transgenic products produced in GM rootstocks can also be intended to change characteristics of the non-GM scion which are relevant for plant breeding, e.g. early induction of flowers to accelerate breeding cycles (ZHANG et al. 2010) or to induce diploidisation of haploid germ cells produced in the scions for reverse breeding (DIRKS et al. 2009).

VOGEL (2016) notes that in addition to the applications described above, the use of GM rootstocks with genetically modified chloroplasts for transgrafting is discussed. With such approaches transgenic plastids may be introduced into different varieties of scions and specifically into plant species whose plastids cannot be transformed yet (VOGEL 2016).

4.10.2 **Experiences and applications generated with Transgrafting**

Grafting was first used in the 4th century BC and it is still used in the large-scale cultivation of plants which are propagated in a vegetative way (e.g. apples, grapes, roses). In recent years also vegetables like tomato, cucumber, melon and eggplant are increasingly grown on rootstocks with improved rooting characteristics (COGEM 2006, SCHAART 2016). Moreover, grafting is widely used for the asexual propagation (i.e. cloning) of commercially grown cultivars.

According to VOGEL (2016) the earliest description of the concept of grafting on GM rootstocks was published in 1991. Testing on genetically engineered rootstock has been carried out on several plant species (references in VOGEL (2016) and (ECKERSTORFER et al. 2014): apple, cherry, grapevine, orange, plum, poplar, walnut, and watermelon. Moreover, the use of genetically engineered rootstocks has been investigated in cucumber, potato, pea, tobacco and tomato (review by (LUSSER et al. 2011a).

Several products have been field tested in the EU according to LUSSER et al. (2011a) and OECD (2016): apple with enhanced rooting characteristics (Sweden), citrange with modified plant architecture (Spain), grapevine with viral resistance (France), pear with enhanced rooting characteristics (Sweden), and fungal resistant orange (Spain). OECD (2016) reports on a walnut with resistance to crown gall disease developed for commercialisation.

The overview on the recent literature retrieved by a survey conducted for the years 2015 until 2017 indicates clearly that a very diverse range of scenarios for use of grafting or transgrafting has to be considered (see Table 15).

As can be seen in the list a wide range of plant species may be used for grafting and transgrafting. The range includes model plants for research (e.g. Arabidopsis and tobacco), annual (crop) species (e.g. tomato, potato, sweet potato, tobacco and rice) as well as perennial plants, in particular fruit trees (e.g. grapevine, cherry, plum, citrus species). Grafting is either involving plant material from the same species, with one or both parts being GM modified, or heterografts with parts from different species, which may be combined through grafting (e.g. bottle gourd rootstocks and watermelon scions, or potato rootstocks and tobacco scions).

Transgrafting approaches found in the literature are not limited to the combination of GM rootstocks with non-GM scions, but include other designs as well (e.g. combinations of different GM rootstocks with GM scions or non-GM rootstocks with GM scions as well as “double grafting”, with three separate components, such as rootstock, lower stem and different upper scion).

In some occasions transgrafting was also used as a tool to induce epigenetic changes in non-GM plant parts, which may result in the generation of traits which are also expressed in subsequent generations of non-GM progeny.

The literature survey also indicates that the method is used for a number of different purposes, including the development of transgrafted plants for potential agricultural application. In such applications transgrafted plants may be used to produce final products, which do not themselves contain genetic modifications. A number of these approaches aim at the induction of disease resistance. However, the results of our survey indicate that a wide range of other traits was targeted (among others tolerance to abiotic stress – e.g. cold, salt, metal stress; plant growth and reproduction traits, e.g. induction of dwarfism, male sterility, early flowering/fruiting and plant composition).

However, the grafting is also used for other relevant types of application including

- the use of transgrafting for the propagation of GM scions on compatible rootstocks to facilitate the production of GM plants from newly transformed plant materials ("micro-grafting", see examples from category number 4 in Table 15),
- the use of GM scions grafted onto appropriate rootstocks to facilitate the rapid generation of plants, which can then be used in the field (for examples see category number 5, Rubio et al. 2015),
- the use of grafting to facilitate the early flowering and fruiting of GM plants to facilitate the production of GM fruit material, e.g. for further testing or propagation. (for example see category number 5, Klementjeva et al. 2016),
- the use of pathogen infected scions as a means to inoculate plants with certain pathogens, e.g. for tests to determine the resistance of specific GM rootstocks or plants to the respective pathogens. Such graft-inoculation is often carried out with pathogen infected buds.
- the use of transgrafting to investigate the systemic dispersal of GM material throughout plants and to investigate the factors influencing the movement of molecules across graft boundaries. For example see ZHANG et al. (2016c), who investigate the RNA motives, e.g. tRNA-like structures, which facilitate long distance transport of the respective RNAs.

A substantial number of the indicated applications of grafting are used to facilitate specific research projects and thus the use of the grafted plants may be limited to propagation under conditions of contained use or in restricted field tests. Such applications may not necessarily lead to unconfined environmental release of the plants. Several of the applications used transgenic reporter gene constructs expressed in the GM part of the grafted plants as tool to address specific research questions.

4.10.3 **Combination of Transgrafting with other approaches**

The survey also retrieved publications which indicate that the use of transgrafted plants is explored as an alternative to the use of whole GM plants, e.g. the application of GM parts (mostly GM rootstocks, but also GM scions) to induce the expression of certain traits in the non-GM part of the transgrafted plants.

Such traits may be used as a tool to facilitate other nGM approaches: Traits which induce early flowering can be used to accelerate breeding cycles (i.e. for accelerated breeding) (ZHANG et al. 2010); traits which induce diploidisation of haploid germ cells produced in non-GM scions can be used to facilitate reverse breeding (DIRKS et al. 2009), and production of specific RNAs which induce specific epigenetic changes in the unmodified part of the transgraft may be used as a tool for RdDM applications (VOGEL 2012).

4.10.4 **Detection and identification of Transgrafting applications**

GM parts of the transgrafted plants (e.g. GM rootstock) can be detected and identified by the same methods as currently applied for the detection and identification of GM plants. However, progeny and products derived from the non-GM parts (e.g. the scion) cannot be detected and identified by such methods (LUSSER et al. 2011a, VOGEL 2012).

Since the vascular systems of GM rootstock and scion are connected, specific products of the transgenic traits (e.g. newly expressed proteins or RNAs) and metabolic compounds resulting from expression of the respective transgenic traits may be transported across the graft boundaries or in general into non-GM parts of the transgrafted plant. The detection of such molecules may be possible by available analytical methods (AGES 2013, KUMAR et al. 2017). However, these methods are not routinely used for such purposes yet and would only allow unequivocal identification of the specific nGM application, if a significant and unique change in the molecular composition of the analysed non-GM plant part is induced by the specific transgrafting application.

If transgrafting is used to induce heritable epigenetic traits in the non-GM parts and non-GM progeny (e.g. via RdDM) the respective epigenetic modifications can be detected analytically. Again such methods are not used routinely for these purposes. In most cases the respective results would not provide unambiguous evidence as required for identification of the material being derived from a transgrafted plant.

4.11 Agroinfiltration

4.11.1 **Concept of Agroinfiltration**

With agroinfiltration techniques genetic material is introduced into plants via bacteria, particularly *Agrobacterium tumefaciens*. In contrast to other techniques using *Agrobacterium* the stable integration of the recombinant DNA is not intended and most applications aim at the transient expression of genetic constructs in plant tissues, mostly in leave tissues (ECKERSTORFER et al. 2014, LUSSER et al. 2011a, VOGEL 2016). The recombinant *Agrobacterium* strain contains the gene to be expressed in the plant inserted into its T-DNA. In cases where *Agrobacterium* contains the foreign gene in a viral vector the method is called agroinfection or agroinoculation. The floral dip method is a specific variant of agroinfiltration, involving reproductive tissue. Using this method flowers are dipped into a suspension of *Agrobacterium* (VOGEL 2016). Floral dip is mostly used to generate stably transformed plants (LUSSER et al. 2011a).

Agroinfiltration may be used to study gene functions as well as for the screening and selection of crop plants with favourable characteristics, for the expression of transgenic proteins (e.g. plant-made pharmaceuticals) or as a tool to facilitate RdDM, or transiently

express transgenes necessary for reverse breeding or genome editing applications using site directed nucleases (ECKERSTORFER et al. 2014).

4.11.2 **Experiences and applications generated with Agroinfiltration**

Agroinfiltration is used since the 1980s, mostly for research purposes. Reviews of examples for early application indicated that agroinfiltration was mainly used in the framework of basic research in model plants, e.g. tobacco (VOGEL 2016).

A survey of the recent literature indicated that agroinfiltration and agroinoculation are mostly used for basic research purposes (Table 16).

According to the literature the research objectives include the study of various plant diseases, the screening for respective resistant plants as well as gene silencing or method development.

Application in plant breeding is also possible, e.g. for the selection of disease resistant plants (VOGEL 2016). Following the agroinfiltration of genes from plant pathogens, the most resistant plants can be selected for breeding (LUSSER et al. 2011a).

A survey by LUSSER et al. (2011a) performed in 2010 indicated, that plant breeding companies use agroinfiltration as a tool in breeding programs for new rape seed, potato and lettuce varieties.

4.11.3 **Combination of Agroinfiltration with other approaches**

Agroinfiltration can be used as a method to facilitate the expression of transgenes in the infiltrated plant parts, as a tool to facilitate other nGM approaches like accelerated breeding, reverse breeding or RNA-dependent DNA methylation (VOGEL 2012).

Agroinfiltration can also be used to introduce constructs for virus-aided gene expression (VAGE) or virus-induced gene silencing into the targeted plants. In the framework of genome editing approaches the method is used to introduce the genetic constructs for expression of the site-specific nucleases as well as templates for SDN-2 and SDN-3, to initiate the intended homologous recombination events in recipient plants.

Floral dip applications, which are used to stably integrate recombinant DNA constructs into plants, without the need for *in vitro* culture of isolated plant cells or plant tissue material are commonly used to generate transgenic plants, but can similarly be used to produce cisgenic or intragenic plants (AGES 2012).

4.11.4 **Detection and identification of Agroinfiltration applications**

Detection and identification of agroinfiltration is only possible transiently in exposed tissues of the treated plants, and not in progeny which do not contain the inserted genetic constructs (LUSSER et al. 2011a, VOGEL 2012).

Detection at the protein level is only possible during their expression (AGES 2012). If stable fragments are inserted detection and identification is possible with similar methods as used for GMOs, provided that the required molecular information concerning the respective inserted sequences is available (LUSSER et al. 2011a).

4.12 CENH3-mediated Haploid Induction (HI)

4.12.1 **Concept of HI**

True breeding lines, i.e. lines with fully homozygous genomes, are highly valuable in plant breeding. Such lines are traditionally generated by inbreeding schemes, which typically require 7-9 breeding steps and therefore a period of several years to obtain a generation with the desired level of homozygosity (BRITT&KUPPU 2016). However, truly homozygous plants can also be obtained from haploid cells via induction of genome duplication to create double haploid (DH) plants within a single generation time (DWIVEDI et al. 2015). The development of haploid gametophytes occurs naturally in some plant species, however for most of the species relevant for plant breeding haploids need to be obtained either by *in vitro* culturing of immature haploid gametophytes, e.g. microspore or anther cell culture (CHEN et al. 2011), or *in vivo* by uniparental chromosome elimination during early development stages of hybrid embryos derived from inter- and intraspecific crosses (DWIVEDI et al. 2015). The latter approach was first developed in barley and since used in a number of other crop species like tobacco, potato, wheat, brassica species and most importantly in maize (DWIVEDI et al. 2015, NIU et al. 2014).

Both *in vitro* and *in vivo* approaches are constrained by technical difficulties: *In vitro* approaches are still not feasible for all species and genotypes, and due to the low rate of embryogenesis and regeneration of plants which may be used further they are time-consuming, labour-intensive and costly (DWIVEDI et al. 2015, SEGUÍ-SIMARRO et al. 2011). Classical *in vivo* approaches are hampered by the limited availability of inducer lines which may be used in crosses leading to formation of haploid offspring and technical difficulties in obtaining the desired double haploid progeny, e.g. associated with a low rate of spontaneous doubling of haploid genomes and technical challenges with the use of chemical agents, like colchicine, for inducing diploidisation *in vitro* (BRITT&KUPPU 2016, DWIVEDI et al. 2015).

A recently developed approach is taking advantage of the important function of the species specific CENH3 histones for chromosome segregation during cell division (BRITT&KUPPU 2016). This histone variant forms centromeric nucleosomes, which play a crucial role in plant development, e.g. for the distribution of chromosomes during cell division. Interference with the association of CENH3 histones with centromeric DNA results in chromosomes, which do not segregate normally in mitotic and meiotic divisions (WATTS et al. 2016). Complete knock-out of CENH3 in plants typically has lethal effects; however such null-mutations may be rescued by CENH3 variants from other species or chimeric CENH3 genes, like the “tailswap” constructs first tested in Arabidopsis (RAVI&CHAN 2010, WATTS et al. 2016).

It was also found in these experiments that complementation of CENH3 knock-outs by tailswap-CENH3 constructs can lead to the induction of haploid formation upon crosses with a mating partner carrying wildtype CENH3 genes (RAVI&CHAN 2010). If a WT male is used for such crosses the maternal chromosomes derived from the CENH3 tailswap parent are eliminated in a highly efficient way, resulting in haploid induction frequencies of up to 40% (BRITT&KUPPU 2016). Further research showed that haploid formation can also be induced in a similar way by certain point mutations in CENH3 genes, which reduce the loading ability of the resulting histone proteins onto centromeres (KARIMI-ASHTIYANI et al. 2015), and complementation of CENH3 knock-outs by CENH3 variants from other species or a variety of different chimeric CENH3 constructs (BRITT&KUPPU 2016, WATTS et al. 2016).

A significant advantage is, that CENH3-mediated haploid induction can also be used with other crop species following approaches developed in Arabidopsis and other model plants (WATTS et al. 2016). First examples of such applications were reported for crop species like maize (KELLIHER et al. 2016), brown mustard (WATTS et al. 2017) and barley (GURUSHIDZE et al. 2017).

Unlike with in vitro approaches, cell culture steps are not required for CENH3-mediated HI and the approach is considered to be applicable for different genetic backgrounds, thus only a single inducer line needs to be established for a specific crop species (WATTS et al. 2016)

HI and the CENH3-mediated HI approach may be particularly useful for crops which do not have a long breeding history and lack an existing repertoire of well characterised homozygous varieties, which can be used for crossbreeding. For such species the rapid generation of true breeding lines is of particular value.

However also for crops used in well-established breeding programmes the CENH3-mediated HI approach can be of significant value: reducing the timespan needed to establish phenotypically advanced, homozygous lines for production of commercial hybrids is regarded to be a crucial aspect for these crops as well (BRITT&KUPPU 2016). In this respect the combined application of CENH3-mediated DH production and marker-assisted selection (MAS) methods have the potential for increasing the speed and precision und thus costs of breeding programmes. Likewise DH technology can also improve the accuracy of genomic selection schemes and speed up the characterisation of qualitative trait loci (SEYMOUR et al. 2012).

If non-GM lines are used as male crossing partner in CENH3-mediated HI, elimination of maternal chromosomes will result in non-GM DH breeding products. However if HI lines with a cytoplasmic male sterility (CMS) background are used, the resulting DH lines will retain the CMS trait. Therefore a straightforward conversion of non-CMS lines into true breeding CMS lines is possible (WATTS et al. 2016).

4.12.2 **Experiences and applications generated with HI**

Experiences with haploid induction initiated by conventionally derived inducer lines, by intra and interspecific crosses and by means of in vitro methods have been gathered in a few plant species for the last 50 years (BRITT&KUPPU 2016). Induction of HI by interference with CENH3-dependent processes in mitosis and meiosis however was first explored by research conducted around 2010 (RAVI&CHAN 2010).

Table 17 is providing an overview on relevant research which was conducted in the years 2011-2017.

The results of the literature survey show that most research relating to CENH3-mediated HI is conducted in model plant species, like Arabidopsis. Such applications rely on the characteristics of modified CENH3 variants generated by GM methods or by TILLING (Targeting Induced Local Lesions in Genomes) approaches based on conventional mutagenesis (Table 17, see Ref. number 1, 2 & 3). TILLING is a method which allows directed identification of different mutations in a specific gene.

Applications of the HI approach based on recombinant CENH3-variants have been developed for maize and brown mustard (see Table 17, Ref. number 9, and 16, respectively).

Other research projects were targeted to adaptation of HI approaches using existing conventional inducer lines for *in vivo* production of doubled haploid plants in different maize cultivars. Other lines of research are dealing with the development of improved protocols for *in vivo* HI in maize and other crop plants, like wheat, and to the development of methods for improved screening of haploid progeny.

4.12.3 **Combination of CENH3-mediated HI with other approaches**

CENH3-mediated HI can in general be implied in all breeding activities where time-consuming back-crossing steps need to be substituted with faster approaches to achieve the same ends.

As regards combination with other nGM approaches CENH3-mediated HI approaches can be used to implement reverse breeding approaches, e.g. as demonstrated in Arabidopsis (WIJNKER et al. 2012).

HI-mediated production of double haploids can also be used to facilitate the production of homozygous lines following application of genome editing approaches (GURUSHIDZE et al. 2017).

4.12.4 **Detection and identification of CENH3-mediated HI applications**

CENH3 inducer lines containing transgenic CENH3 variants may be detected and identified by existing methods for GMO detection, particularly when these approaches can be based on knowledge concerning the integrated transgenic constructs.

Identification of the non-transgenic products derived from application of CENH3-mediated HI is virtually impossible by analytical means only, without additional information from documentation systems supporting traceability of the origin of particular breeding products.

4.13 Reverse Breeding

4.13.1 **Concept of Reverse Breeding**

Reverse breeding is applied to facilitate the consistent reproduction of specific heterozygous (hybrid) elite lines, which are selected for their phenotypic characteristics. When such heterozygous lines are not established in a deliberate breeding strategy using homozygous parental lines, but only selected from a mixed field population for their superior phenotypic characteristics, it is not possible to further produce plants with a similar heterozygous genotype. If the appropriate homozygous parental lines, which are required for this purpose are not available beforehand, they cannot be established by traditional breeding methods involving further crossbreeding, since the meiotic recombination events which occur during sexual reproduction would disrupt the specific allele combinations present in the selected hybrid line (WIJNKER&DE JONG 2008). The main advantage is that phenotypically superior heterozygous plants can be propagated without prior knowledge of their genetic constitution. Reverse breeding therefore would be particularly valuable for plant breeding purposes in species which are not sufficiently genetically characterised and for which no extensive collection of characterised true breeding lines exist (DIRKS et al. 2009).

Reverse breeding is an approach for the construction of homozygous lines which preserve the allelic combinations of the selected hybrid plant (DIRKS et al. 2009). Reverse breeding consists of several steps, the first step is to genetically modify the selected hybrid plant to suppress meiotic recombination during production of haploid gametes in these plants. In a second step haploid gametes derived from the modified plant are converted into double haploid offspring, e.g. by approaches as described in Chapter 4.12 above. For a proof of concept study in Arabidopsis a CENH3-mediated approach for chromosome elimination was used to produce such DH lines (WIJNKER et al. 2012). These plants are then screened to identify a pair of homozygous lines that reconstitutes the genotype of the selected heterozygous plant upon crossing (DIRKS et al. 2009). In addition only homozygous lines are used, which do not carry the previously introduced transgenic modification. This pair of homozygous lines can then be used subsequently as parental plants to produce seed material for the production of the original hybrid elite line (WIJNKER et al. 2012). Due to methodological constraints the method is limited to crops with a haploid genome consisting of 12 or less chromosomes (LUSSER et al. 2011a).

According to VOGEL (2016) this method can also be used for the production of chromosomal substitution lines for further breeding (where one parental plant contains also chromosomes of the other parent and *vice versa*) or the conservation of elite genotypes (including uncharacterised heterozygous genotypes).

4.13.2 **Experiences and applications generated with Reverse Breeding**

Reverse breeding was first proposed as a solution to the challenge of fixation of complex heterozygous genomes by constructing complementing homozygous lines in 2003, and the concept was further elaborated in 2009 (DIRKS et al. 2009).

A proof of principle study conducted in Arabidopsis was published in 2012 (WIJNKER et al. 2012).

However, applications of the technique in major crop plants are not described in OECD (2016) or other reviewed literature.

A variant of the approach termed marker-assisted reverse breeding (MARB) was developed in maize (GUAN et al. 2015). However MARB is different to the original concept of RB in a number of aspects: it does not involve RNAi-based silencing of meiotic recombination, it is not limited to plant species with a limited number of chromosomes like RB, but it is heavily dependent on the availability of high density genotyping for the particular crop species.

4.13.3 **Combination of Reverse Breeding with other approaches**

Reverse breeding can be used in combination with transgrafting, VIGS or centromer-mediated genome elimination. AGES (2013) lists RNAi or VIGS as possibilities for combination as well as ZFN, ODM and RdDM techniques. However, it is stated that there are no indications for respective applications in the near future.

4.13.4 **Detection and identification of Reverse Breeding applications**

Products based on reverse breeding are free of extracellular introduced DNA sequences, since transgenes are used only in the intermediate breeding steps. Therefore, detection and

identification is not possible. Those products cannot be distinguished from varieties based on conventional hybrid breeding (AGES 2013, LUSSER et al. 2011a, SCHUTTELAAR 2015, VOGEL 2012).

5 Considerations for the environmental risk assessment of nGM applications

5.1 General considerations for the risk assessment of nGM plants

An important issue in the framework of the general discussion on nGMs at the political level is to define the specific biosafety issues relating to the various nGMs and their applications in plant breeding. Therefore an objective of the study at hands is to provide considerations, which risk issues need to be considered for specific nGM applications.

To address this issue it needs to be determined, whether specific nGMs or the traits developed with such nGMs might be associated with a potential for specific adverse effects. The results of such an analysis also need to be considered for the development of suitable approaches for the risk assessment of nGM applications and products derived from them.

In a report addressing among others risk assessment issues for genome editing applications only the effects of unintended genetic modifications due to the characteristics of the used nGM methods are considered (BVL 2017). However, the report of the chief scientific advisors to the EU commission “New Techniques in Agricultural Biotechnology” (SAM 2017) recommends an appropriate approach for the risk assessment of environmental effects needs to consider all of the following issues:

- Effects due to intended changes
- Effects due to unintended changes
- Characteristics of the modified plant species
- Characteristics of the receiving environment
- Intended use of the modified plant and exposure of the environment

The issues relevant for a comprehensive risk assessment are addressed in the following chapters. Trait-related and method-related considerations for risk assessment are discussed separately in the following chapters.

5.1.1 **Trait-related considerations**

Some aspects can only be discussed in relation to the specific characteristics of individual modified plants (i.e. in relation to the plant species, the developed trait and the intended use in agriculture which is determining the environmental exposure to the modified plant). This part of the discussion therefore needs to take into consideration the specific breeding goals which are pursued by application of different nGMs and is based on the range of examples for specific applications of the different nGMs found in the surveyed literature. This range comprises nGM plants which have already been developed for specific agricultural purposes as well as reports concerning ongoing research and development projects. Examples for both types of applications were presented in the previous chapters addressing experiences with and applications generated by specific nGMs and can be clustered into groups combining plants with related traits, which may cause comparable environmental effects:

- Herbicide resistant (HR) plants
- Disease resistant (DR) plants

- Plants with altered composition
- Plants with resistance to environmental stress factors

Since the grouping is based on the intended traits rather than on the nGM used for development these groups comprises of plants modified by all different nGMs, which are or may be used to develop the respective traits. Also the direct or indirect environmental effects resulting from these plants will mostly be determined by the nature of the different developed traits and the intended use of such plants.

An assessment of trait-related environmental effects must also address the question whether unintended effects might be associated with the particular modifications in addition to effects of the intended phenotypic of the respective trait.

5.1.2 **Approach to identify potential environmental effects associated with different groups of traits**

The following questions were considered for either of the above outlined groups of nGM plants.

- Which plant species was modified to develop the intended trait?
- Which genetic components (gene(s) or regulatory genetic elements) are modified to generate the trait?
- In which mechanism(s) at the cellular and plant level are the modified genetic components involved?
- Were comparable traits established by conventional breeding or GM technology for similar agronomical use?
- Which direct and indirect adverse effects (environmental and health effects) may be associated with the traits (including pleiotropic effects associated with the trait)?

For the latter issue the following information can be considered relevant to identify potential adverse effects associated with nGM plants:

- Risk hypotheses addressed in a risk assessment of comparable GM plants;
- Risks identified in the course of the risk assessment of (GM) plants with similar/comparable traits, including information on risk management requirements for such plants;
- Information on (environmental) effects, which were identified upon use of (GM) plants with similar/comparable traits

The assessment of environmental effects may be facilitated by information on the following characteristics of the traits contained in respective nGM plants

- Stability of the trait
- Expression characteristics of the trait (Where? When? Level of expression?)
- Heritability of the trait

5.1.3 **Method-related considerations**

For a comprehensive risk evaluation however method-related considerations need to be taken into account in addition to trait-based considerations. Method-related considerations are particularly relevant for an assessment of any unintended effect associated with nGM applications. Method-related unintended molecular changes may be associated with different aspects of the overall development process of nGM products. They either depend on the mechanisms of the used nGM itself or on the characteristics of further methods: e.g. methods which are required for the overall development of a particular nGM plant, like methods for *in vitro* cultivation of plant cells and tissues or methods for regeneration of plants from these cultures. For some nGMs GM methods are used to introduce and express the molecular tools initiating nGM processes in the recipient plant cells. Other nGMs, like cisgenesis, intragenesis or transgrafting, are comparable to transgenesis as far as the methods to introduce the respective genetic constructs into the genome of plant cells are concerned.

Like GM technology the presently available nGMs, are not fully specific to introduce only the intended molecular changes into plants. Thus a range of unintended molecular changes may be introduced by the particular nGM method and they can lead to phenotypic effects affecting the properties of the modified plant (SAM 2017). According to the nature of these particular phenotypic effects, the unintended changes may be considered either harmless or adverse regarding human health and the environment.

The approach for the assessment of unintended adverse effects therefore needs to take into account two layers of considerations:

- technique-related considerations regarding unintended molecular changes associated with breeding approaches using different nGMs
- considerations whether such molecular changes may result in adverse effects (environmental effects and health effects

5.1.4 **Approach to identify unintended effects associated with different nGM methods**

For a comprehensive evaluation of potential unintended effects the assessment should not focus on the characteristics of a specific step of an overall nGM approach alone. On the contrary: All steps which are necessary for the development of an nGM plant need to be considered to address effects associated with the application of the different nGM methods. This involves also method-related considerations related to other involved procedures, including the application of other nGM techniques as methodological tools, any modifications by GM methods introduced at intermediary steps or any other (biotechnological) procedures which are involved in the respective work-flows for the development of particular nGM plants.

The following issues are relevant for the characterization of method-related effects:

- Aspects related to the introduction of method components and to genetic transformation of the recipient cells, if necessary
    - Which type of delivery system is used for introducing the molecular components for an nGM? (stable GM transformation, transient introduction of

expression constructs, introduction of preassembled, active components, like proteins, riboproteins or oligonucleotides)

- How long are method-related constructs/components present?
- Which type of genetic transformation system is used? (e.g. *Agrobacterium*-mediated methods, biolistic transformation, PEG-mediated transformation, etc.).
- Which (mutational) effects are associated with the used delivery or transformation method?
- Which positional effects may result from the genetic modifications?
- Are transgenic constructs stably inserted? (Which effects are due to integration of such constructs?)
- To which extent are transgenic constructs removed during later steps?

- Aspects related to nGM technologies designed to introduce site-specific mutations
  - Are off-target effects possible with the nGM(s) involved? (Which kind of off-target effects are known and can such off-target effects be detected?)
  - Which unintended molecular changes can happen at the genomic target site?
  - Which unintended molecular changes can happen at the off-target sites? (Including deletions, insertions, genetic rearrangements, mobilisation of transposable elements, etc.)?
  - Which adverse effects may result from unintended changes? (e.g. toxicological or allergenic effects, pleiotropic effects)
- Aspects related to further molecular changes associated with nGMs
  - Which epigenetic changes may be associated with genetic modifications?
  - How stable are the induced genetic and epigenetic changes?
- Aspects related to other biotechnological methods used for development of nGM applications
  - Which mechanism is used for selection of modified individuals?
  - Which (biotechnological) methods for tissue or cell culture are used for transformation and regeneration?
  - Are protoplast-based methods used?
  - Are substances with mutagenic or hormonal effects used for culturing cells?
  - To which extent are somatic mutations introduced during (cell) culture eliminated by the breeding process?

5.2 Risk issues associated with nGM plants expressing different types of traits

5.2.1 Environmental effects associated with herbicide resistant (HR) nGM plants

HR traits are introduced into nGM plants for several reasons: On the one hand HR traits are used as selectable markers to screen for successful applications of methods and as tools for the improvement of particular GM methods itself (CARVALHO&FOLTA 2017, KONAGAYA et al. 2013, KUMAR et al. 2016). On the other hand HR traits and combinations of HR traits are developed in nGM plants for use as a tool for weed control in agricultural production (CHAUHAN et al. 2017, D'HALLUIN et al. 2013). This chapter focuses on applications of the latter type, which are developed with a view to future practical use in agriculture.

nGM applications with HR traits

Different HR traits were developed by a number of nGM approaches, specifically traits conferring resistance to inhibitors of acetolactate synthase (ALS) genes (e.g. imamazamox, chlorsulfuron), enolpyruvylshikimate-3-phosphate synthase genes (e.g. glyphosate), glutamine synthetase (e.g. glufosinate), hydroxyphenylpyruvate dioxygenase (HPPD) (e.g. tembotrione) and acetyl CoA carboxylase (ACCase) (e.g. quizalofop). Resistance is due to different mechanisms and is conferred towards a variety of different herbicide classes and substances, as illustrated by the following examples:

- ALS-inhibiting herbicides target the enzyme acetohydroxyacid synthase (AHAS) – also called acetolactate synthase (ALS), which is essential for the biosynthesis of branched-chain amino acids (i.e. valine, leucine and isoleucine). Five different chemical families of ALS-inhibiting herbicides are widely used: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB) and sulfonylamino-carbonyl-triazolinones.
- The glyphosate-based herbicides are inhibitors of 5-enolpyrovylshikimat-3-phosphatsynthase (EPSPS)-inhibiting herbicides. This class of herbicides competes with the substrate phosphoenolpyruvate (PEP) at the EPSPS enzyme-binding site in chloroplasts and inhibits the synthesis of essential aromatic amino acids (tryptophan, tyrosine and phenylalanine) via the shikimate pathway.
- Glufosinate-ammonium (GA), another widely used non-selective herbicide, inhibits the glutamine synthetase, an enzyme which plays a central role in the detoxification of ammonia, which is formed during various metabolic processes in the plant cell. This herbicide interferes with the nitrogen metabolism in plants leading to accumulation of toxic nitrogen compounds in the affected plant tissue, resulting in withering and necrosis of plant tissues (Freyssinet M. 2002).

Different mechanisms for resistance are exploited to engineer traits which confer resistance towards these herbicides.

An approach which is also employed for the development of HR plants is based on the metabolic detoxification of the respective herbicides such as glyphosate, glufosinate and 2,4-D in modified plants. This is commonly achieved by the transfer of heterologous (microbial) genes into plants via GM or nGM methods and their expression in the modified plants to degrade or chemically modify the respective herbicidal compounds (KANDASAMY O.S. 2002).

The PAT enzyme for example, expressed by either the *bar* or the *pat* gene, specifically acetylates phosphinothricin, i.e. glufosinate-ammonium. This is inducing a degradation process during which various metabolites are formed, which do not have herbicidal effects (FREYSSINET M. 2002).

Other resistance mechanisms are based on the alteration of the (enzymatic) target site, where herbicides take effect (target-site resistance). A prominent example for the latter is the development of resistance against ALS-inhibiting herbicides. Here resistance to the herbicide is a result of mutations in the endogenous ALS genes, which reduce the sensitivity of the AHAS enzyme expressed in the plant to this class of herbicides. The specific AHAS enzyme mutants differ with respect to the tolerance they confer to various ALS inhibiting herbicides (GREEN 2007, TAN et al. 2005). Particular alterations in the targeted ALS genes do not automatically confer cross-resistance to all ALS-inhibiting herbicides. While some mutants are exclusively tolerant to imidazolinone herbicides, others are cross tolerant to other ALS inhibitors (e.g. sulfonylureas) (TAN et al. 2005). The level of resistant is dependent on the both the mutation type and the zygosity of the trait (TAN et al. 2005). Resistance of this type can be developed by transfer of mutated copies of the respective ALS genes into a plant by GM or nGM methods such as intragenesis, cisgenesis or SDN-3 applications of genome editing. In an alternative approach the endogenous target genes may be altered by different approaches of mutagenesis, including approaches for genome editing by SDN or ODM methods aiming to introduce either random (SDN-1) or directed sequence changes (SDN-2 or ODM) into the respective plant genes, such as the ALS genes.

Table 2 lists a number of different nGM applications with HR traits. These applications are in different stages of development – from early research and development to testing for commercial use in agriculture. The herbicide resistant oilseed rape developed by Cibus using ODM is the only HR nGM plant which is actually cultivated so far, but other crops with HR traits are in the commercial pipeline. It can be expected that herbicide resistant nGM crops will continue to be an important objective for future commercial plant development (KASKEY 2018).

Table 2: Overview of plants with herbicide resistance traits developed by nGMs

nGM approach	**Plant**	**Specific type of nGM used**	**Molecular mechanism**	**Literature**
Genome Editing (by SDNs)	Potato	SDN-1 TALEN	Mutations induced in the endogenous ALS gene to generate resistance to ALS-inhibitor herbicides	(NICOLIA et al. 2015b)
	Potato	SDN-1 CRISPR/Cas9	Introduction of modifications of the endogenous ALS1-genes present in elite potato lines to generate crops with resistance to ALS-inhibiting herbicides	(BUTLER et al. 2015)
	Potato	SDN-2 TALEN	Introduction of modifications by transient expression of TALENs to the endogenous ALS1-genes present in elite potato lines to generate crops with resistance to ALS-inhibitor herbicides	(BUTLER et al. 2015)

	Potato	SDN-3 (targeted integration of transgenes) TALEN	Targeted insertion of a promoter-less ALS-gene into a predefined genomic locus (endogenous Ubi7 locus) to generate resistance to ALS-inhibiting herbicides	FORSYTH et al. (2016)
	Tobacco	SDN-1 ZFN	Mutations induced in endogenous ALS genes	(TOWNSEND et al. 2009)
	Tobacco	SDN-3 ZFN	Method for gene replacement or multigene exchange that could also be applied in a context of commercial development (using kanamycin resistance and bialaphos herbicide resistance as test case)	(SCHNEIDER et al. 2016)
	Rice	SDN-2 (gene replacement) TALEN	Genomic ALS gene replaced by ALS gene fragment with two independent point mutations to generate resistance to ALS-inhibiting herbicides	Li et al. (2016) IN SUN et al. (2016a)
	Soybean	SDN-1 CRISPR/Cas9	Generation of a chlorsulfuron resistant soybean by modification of the endogenous ALS1 gene using transient expression of CRISPRreagents	(BUTLER et al. 2016)
	Cassava	SDN-3 CRISPR/Cas9	Approach to insert mutated copy of EPSPS gene at the native cassava locus to generate non-transgenic cassava resistant to glyphosate-based herbicides	(CHAUHAN et al. 2017)
	Cotton	SDN-3 MN	Insertion of additional herbicide resistance genes (HPPD, EPSPS) by trait stacking in cotton through targeted double-strand break induction	(D'HALLUIN et al. 2013)
	Maize	SDN-2/3 ZFN	Approach to create a trait stacking system via intra-genomic homologous recombination, employing 'trait landing pads' (TLPs) to generate maize with glyphosate-resistance	(KUMAR et al. 2016)
	Maize	SDN-3 ZFN	Approach to simultaneously exchanges a selectable marker gene while integrating additional transgenes, such as the glufosinate herbicide resistance gene, during the transformation process	(KUMAR et al. 2015)

	Maize	SDN-3 ZFN (EXZACT)	Approach to create modular 'trait landing pads' (TLPs) for subsequent targeted transgene integration and trait stacking in crop plants. The pre-inserted PAT herbicide resistance gene provided the target sequence for subsequent SDN3-mediated transgene integration of the AAD1 herbicide resistance into maize.	(AINLEY et al. 2013)
Genome editing (by ODM)	Oilseed rape	SDN-2 ODM	Generation of targeted mutations using the Cibus RTDS™ system to generate resistance to sulfonylurea herbicides. SU Canola is authorized in CAN and marketed in the USA	(GOCAL et al. 2015)
	Flax (*Linum usitatissimum*)	SDN-2 Combination of ODM with SDNs (TALEN, CRISPR)	High-frequency generation of glyphosate-resistant plants by ODM-based modification of the EPSPS gene	(SAUER et al. 2016b)
	Flax	SDN-2 ODM	Generation of targeted mutations using the RTDS system to generate resistance to glyphosate-based herbicides.	Abbot et la. 2015
	Rice	SDN-2 ODM	Generation of targeted sequence modifications in the ALS gene by ODM to generate resistance to ALS-inhibitoing herbicides.	Abbot et la. 2015
Intragenesis	Strawberry	-	Intragenic approach based on overexpression of the native FvEPSPS gene to develop transformation vectors for strawberries	(CARVALHO&FOLTA 2017)
Cisgenesis	Chinese cabbage	-	Use of the ALS gene as a cisgenic selectable marker in Chinese cabbage	(KONAGAYA et al. 2013)

Many different HR traits are also commercialised in GM crop plants, an overview is provided in Table 3 and in KOHL (2018). Some of these HR traits are comparable to the ones developed in nGM plants in Table 2.

Table 3: Overview of HR traits commercialized in GM crops (source: ISAAA GM approval database: http://www.isaaa.org/gmapprovaldatabase/default.asp

Herbicide	**HR genes (applied in various combinations)**	**Function**	**Plant**
2,4-D	*aad-1*	Detoxifies 2,4-D herbicide by side-chain degradation and degrades the R-enantiomers of aryloxyphenoxypropionate herbicides	maize, soybean
dicamba	*dmo*	Confers tolerance to the herbicide dicamba by using dicamba as substrate in an enzymatic reaction	maize
glufosinate	*bar*	Eliminates herbicidal activity of glufosinate (phosphinothricin) herbicides by acetylation	OSR, chicory, cotton, maize, soybean, rice
	pat	Eliminates herbicidal activity of glufosinate (phosphinothricin) herbicides by acetylation	OSR, cotton, maize, soybean, sugar beet
glyphosate	*gat4621*	Catalyzes the inactivation of glyphosate, conferring tolerance to glyphosate herbicides	OSR
	cp4epsps (aroA:CP4)	Ddecreases binding affinity for glyphosate, thereby conferring increased tolerance to glyphosate herbicide	OSR, cotton, creeping bentgrass, maize, potato, soybean, sugar beet, wheat
	goxv247	Confers tolerance to glyphosate herbicides by degrading glyphosate into aminomethylphosphonic acid (AMPA) and glyoxylate	OSR, maize
	2mepsps	Decreases binding affinity for glyphosate, thereby increasing tolerance to glyphosate herbicides	cotton
	mepsps	Confers tolerance to glyphosate herbicides	maize
	gat4601	Catalyzes the inactivation of glyphosate, conferring tolerance to glyphosate herbicides	soybean
mesotrione	*avhppd-03*	Tolerance to mesotrione herbicides	soybean
oxynil	*bxn*	Eliminates herbicidal activity of oxynil herbicides (eg. bromoxynil)	OSR, cotton
sulfonylurea	*surB*	Confers tolerance to sulfonylurea herbicides and other acetolactate synthase (ALS-) inhibiting herbicides	carnation, maize
	als	Allows the synthesis of essential amino acids in the presence of sulfonylurea herbicides	flax
	csr1-2	Confers tolerance to imidazolinone herbicides	soybean
	gm-hra	Confers tolerance to applications of sulfonylurea -based herbicides	soybean

HR traits were also developed by conventional breeding methods. In particular the Clearfield® system by BASF has successfully been marketed in five different crop plants

since the Nineties (i.e. maize, OSR, rice wheat and sunflower). This HR system combines ALS-inhibiting herbicides and imidazolinone-tolerant crop plants. In Europe the Clearfield® system has so far been introduced for OSR with three complementary herbicides available: Clearfield®-Vantiga® D, Clearfield®-Vantiga® D Runway™-Pack and the metazachlor-free Clearfield®-Clentiga®. Clearfield® sunflower can be used with the complementary herbicide Pulsar® Plus (active ingredient: imazamox). In addition Pioneer is offering the DuPont™ Express Sun® system for Sunflowers which are resistant to the sulfonylurea herbicide DuPont Pointer® SX® (active ingredient: Tribenuron) (Pioneer Homepage https://www.pioneer.com/web/site/germany/oilseed/sunflower/).

All commercialised crops of the Clearfield® system have been developed by selection and mutagenesis (of seed, cell culture or pollen) except for sunflowers marketed in the US, which was developed from an imazethapyr-tolerant wild sunflower population discovered in the US (TAN et al. 2005). Imidazolinone-tolerant mutants have also been discovered in sugar beet, cotton, soybean, lettuce, tomato and tobacco (TAN et al. 2005).

Risk issues for nGM plants with HR traits

Experience with effects resulting from HR traits is available from existing risk assessments of herbicide resistant GM plants. Of particular relevance are indirect effects on biodiversity resulting from the changes in weed management, and the development of herbicide resistant weeds (EFSA 2010, SCHÜTTE et al. 2017). For herbicide resistant oilseed rape, experience is available from comparable conventional HR crops, which raised a number of concerns, e.g. dispersal and persistence of HR volunteers (EXPERTGROUP 2014, HUANG et al. 2016).

Potential adverse effects of HR GM plants were addressed during the risk assessment of a number of notifications for the authorization of HR GM plants in the EU, which included the scope of cultivation. Such effects are also considered in the EFSA Guidance document on ERA (EFSA 2010, p.77 ff) and in the scientific literature (DEVOS et al. 2008, SCHÜTTE et al. 2017). Of particular relevance, among others, are the following issues:

- GM HT crop volunteers (especially volunteer OSR) and weed relatives, which acquire the trait by plant-to-plant gene transfer, may require additional measures for control in other crops (e.g. use of specific herbicides) and result in additional environmental impact, in particular if these weeds are resistant to multiple herbicide.
- The selection pressure exerted by repeated applications of the same herbicide across crop rotations will eventually lead to the development of resistant weeds and changes in weed community diversity due to weed shifts.
- The effects of the altered weed management system (fewer weeds and/or weed shifts) in GM HR crops may affect biodiversity (flora and fauna) in and around fields (Firbank 2003).
- In some cases (GM HR soybean) the GM HR crop herbicide management may have effects on the nitrogen-fixing symbionts (e.g. *Bradyrhizobium japonicum*) and result in changes in the soil microbial communities and changes in the use of nitrogen fertilizer (EFSA 2012).

Such effects are not specific to GM HR plants. As noted by ISHII&ARAKI (2017) ALS-resistant rice which was cultivated in Italy and the USA hybridized with related wild species and HR

resistant weeds emerged from these outcrossing events. This underlines the fact that the assessment of the herbicide resistance traits is important independent of the method or technology that was used to produce the respective crops.

Some GM crops, in particular soybean, have been made resistant to multiple herbicides, including glyphosate, glufosinate ammonium, dicamba and others[8]. Such crops can be expected to contain mixes of different pesticide residues. When methods to assess the cumulative and synergistic effects of pesticides are developed (EFSA 2013, PRODUCTS&RESIDUES 2013), they have to be taken into account for risk assessment and potential human health impacts (Regulations (EC) No. 396/2005 and No. 1107/2009). A report by the European Food Safety Authority (EFSA) further details how to consider effects of pesticide cocktails on the nervous system (AUTHORITY et al. 2019). As genome editing approaches based on SDN-2 and SDN-3 technology are available for the targeted introduction of (multiple) herbicide resistance genes, e.g. resistance to glufosinate ammonium and 2,4 D herbicides in maize generated with ZFN-based approaches (AINLEY et al. 2013), cocktail mixes of pesticide residues can be expected to become a relevant risk assessment issue for HR nGM crops as well.

In addition to effects connected to potential changes in herbicide regimes (e.g. type of herbicide used and timing of herbicide applications) and the changes in cultivation management (e.g. adoption of minimum tillage or no-tilling cultivation techniques), other risk issues also need to be considered: It has recently been shown in Arabidopsis that elevated expression levels of modified EPSPS can lead to pleiotropic effects, like elevated auxin content and increased fecundity of the modified plants (FANG et al. 2018). Therefore the absence of unintended effects on composition or other phenotypic properties should be confirmed for HR nGM crops.The risk assessment of GM HR crop plants conducted in the EU identified potential adverse environmental effects which are either directly or indirectly associated with the use of herbicides and the resulting changes in cultivation management. Consequently risk mitigation and management – including their evaluation – is considered necessary. This is in line with results of the technology assessment conducted in Germany for the Clearfield® system, which identified the need for mitigation measures to avoid negative effect in particular cropping systems due to the dispersal of the resistance traits (in volunteers and wild relatives), the increase in resistance development in weeds and the expected increase in herbicide use (EXPERTGROUP 2014).

5.2.2 **Environmental effects associated with disease resistant (DR) nGM plant**

DR traits have been developed for many different crops by using a range of different nGMs (see Table 4). DR traits are developed in plant species traditionally used for research purposes (e.g. *Arabidopsis thaliana*) as well as in commercially relevant species: (i) vegetables, like cucumber, tomato, potato, grapevines, tobacco, (ii) trees, like citrus, apple, orange, peach and plums, and (iii) walnuts as well as (iv) cereal crops such as rice and wheat.

[8] http://bch.cbd.int/database/lmo-registry/

DR traits are developed to increase resistance towards different pathogens, including plant viruses, bacterial and fungal pathogens (see Chapter 5.2.2). A number of different approaches are pursued:

- Use of nGM approaches such as genome editing to disrupt host susceptibility factors for pathogen infection and development of the respective plant.
- Expression of new resistance factors (genes) in the target plants following insertion of expression constructs by cisgenesis or intragenesis.
- Use of transgrafting to facilitate the silencing of critical pathogen functions, e.g. by RNAi-mediated by interfering RNAs expressed in GM rootstocks.

nGM plants with resistance to plant viruses

Approaches applied to achieve virus resistance in plants include targeted modification of viral host factors and host factor genes and RNA silencing approaches targeting viral proteins. Commercially important viral diseases caused by potyviruses, potato virus Y, tobacco or cucumber mosaic virus, tomato yellow leaf curl virus, citrus tristeza virus as well as plum pox virus have been targeted. Virus resistance has been achieved by the use of approaches for genome editing like SDN-1 (CRISPR/Cas 9), intragenesis or transgrafting (Table 4).

For potyvirus resistance in *Arabidopsis thaliana* CRISPR/Cas9-based genome editing was used to introduce a sequence specific point mutation into a target sequence present in a viral host factor (translation initiation factor eIF) which is required for viral survival (PYOTT et al. 2016). Eukaryotic translation initiation factors (eIF) play a key role in the host protein translation in eukaryotes and interact with viral proteins (VPg). It has been shown that different potyviral VPg bind specifically to different isoforms of eIF, which is necessary for successful virus infection and translation, replication and cell-to-cell movement of the virus genome (CAVATORTA et al. 2011, PYOTT et al. 2016). The specific point mutation at the eIF(iso)4E locus leads to a loss of gene function and consequently to complete resistance of Arabidopsis plants to turnip mosaic virus (PYOTT et al. 2016). Other efforts to induce potyvirus resistance in cucumber targeted an eIF gene by introducing deletions and single nucleotide polymorphisms (CHANDRASEKARAN et al. 2016). Virus Y resistance in potato was generated by expression of a mutated resistance gene for an eIF by site-directed mutagenesis (CAVATORTA et al. 2011). The overexpression of a novel variant of the eIF with a range of amino acid substitutions conferred virus resistance in wild *Solanum* species (DUAN et al. 2012).

Another method was applied in order to induce sharka-virus resistance in plum trees (*Prunus domestica*) by grafting transgenic plum pox virus (PPV) resistant buds onto virus free rootstocks of plum (GARCÍA-ALMODÓVAR et al. 2015, POLÁK et al. 2017, SIDOROVA et al. 2016). The applied methods are based on a RNA silencing mechanism involved in the naturally occurring defence mechanisms against viruses. Transgenic plants were created which express pathogen-derived sequences encoding RNAi inducing hairpin RNAs. These RNAs are inducing the silencing of the viral P1 and HC-Pro viral proteinase genes which, beside the cleavage of the viral genome, prevent the expression of anti-silencing proteins. The transgenic lines were then grafted onto PPV infected plants (GARCÍA-ALMODÓVAR et al. 2015). Also the viral coat protein of the PPV was engineered into transgenic plants. The transgenic plant did not express the PPV coat protein, but low levels of the mRNA were

detected in the cytoplasm. The underlying mechanism in the transgenic plants was therefore considered to be based on post-transcriptional gene-silencing by short interfering RNA (siRNA) in the plants. These siRNAs control the viral RNA silencing (see POLÁK et al. (2017) and references therein).

nGM plants with resistance to bacterial pathogens

Some of the approaches to induce resistance to bacterial pathogens are targeting plant susceptibility genes (S genes). Absence of S genes confers resistance to the specific pathogen, e.g. by loss-of-function. Secretion of pathogen effectors plays a crucial role in plant-microbe interactions. If the effector targets play a negative role in plant defence, then knocking out this effector target leads to resistant plants (see review in PAVAN et al. 2010). Resistance to bacterial diseases such as citrus canker or greening disease, bacterial blight diseases in rice has been developed by targeting S genes using SDN-1 techniques with CRISPR/Cas9 and TALEN (Table 4).

Resistance to the bacterial pathogen *Xanthomonas citri* causing canker in grapefruit was achieved by modifying the coding region of the susceptibility gene CsLOB1 by CRISPR/Cas9, resulting in 1bp insertions or 2-22 bp deletions in the target alleles. The CsLOB1 susceptibility gene is a plant transcription factor. The bacterial pathogen (*X. citri*) encodes TAL effectors recognizing the promoter of the CsLOB1 gene and inducing expression of the susceptibility gene (see JIA et al. (2016a) and references therein). The recognizing region of the promoter is an effector binding element (EBE) region and is therefore critical for susceptibility to the pathogen (JIA et al. 2016a).

Rice resistant to the bacterial pathogen *Xanthomonas oryzae* was generated by inducing mutations in susceptibility genes by genome editing via CRISPR/Cas9, TALEN approaches (LI et al. 2012). In modified plants the virulence functions of the pathogen`s effectors are disrupted without affecting the developmental function of the Os11N3 gene (LI et al. 2012). In non-modified plants pathogens activate the susceptibility gene Os11N3 which allows them to use the plant's cell sugars to satisfy the pathogen's nutritional needs. Here also an effector-binding element (EBE) in the susceptibility gene`s promoter region is relevant for the pathogen's effector` action. Also another gene OsSWEET13 was identified as a disease susceptibility gene in rice (ZHOU et al. 2015).

Other approaches were based on the expression of antimicrobial proteins as defence strategy against plant pathogens. Resistance to citrus canker and another commercially important bacterial disease of citrus (Huanglongbing or citrus greening disease) was reported by overexpression of a plant thionin in a transgenic citrus plant (HAO et al. 2016). As endogenous thionin levels are not sufficient for pathogen protection, a synthetic thionin gene was synthesized and introduced to create transgenic plants. Thionins are cysteine-rich peptides with antibacterial, antifungal and other activities. They are considered to induce the opening of cell membrane pores of pathogens which results in loss of ions from the cells (HAO et al. 2016). Enhanced disease resistance in the modified Citrus plants most likely based on growth inhibition of the pathogen *Xanthomonas citri* was shown by grafting of pathogen-infected buds on the transgenic plants which would result in spreading of the disease through unprotected non-GM plants (HAO et al. 2016).

Another approach was based on silencing of *Agrobacterium tumefaciens* oncogenes which induce Crown Gall disease in walnut (ESCOBAR et al. 2002). Expression of inducers of post-

transcriptional gene silencing homologous to the oncogenes resulted in degradation of the corresponding oncogene mRNA transcripts *in planta* thereby achieving resistance (ESCOBAR et al. 2002).

nGM plants with resistance to fungal pathogens

Resistance to fungal diseases such as powdery mildew (wheat, tomato, grapevine), late blight (rice, potato), black spot disease in potato, scab in apples by NBT was so far induced via SDN-1 (CRISPR/Cas9 and TALEN), cisgenesis and transgrafting approaches (Table 4).

One approach was based on targeted knock-out of a negative defence regulator suppressing plant defence (PAVAN et al. 2011). The MLO protein negatively regulates disease resistance to powdery mildew in different plants. The *mlo* locus is known to encode membrane-associated proteins and confers susceptibility to fungi causing the powdery mildew disease (ACEVEDO-GARCIA et al. 2014). Loss-of-function mutations in the *Mlo* allele lead to resistance (PAVAN et al. 2011). A tomato variety resistant to powdery mildew *Oidium neolycopersici* was achieved by a loss-of-function mutation (deletion of 48 bp) in one *mlo* locus (simlo1) using the CRISPR/Cas9 system (NEKRASOV et al. 2017). Similarly, TALEN-induced targeted simultaneous mutations in three *mlo* alleles were used to establish powdery mildew resistance in wheat (WANG et al. 2014a).

Resistance to potato late blight caused by the fungus *Phytophthora infestans* was achieved by transferring several resistance genes from wild *Solanum* species to commercially used potato varieties of *Solanum tuberosum* (HAVERKORT et al. 2016). Marker-free transformation and selection of events free of vector backbone sequences ensured the selection of truly cisgenic plants (HAVERKORT et al. 2016). The late blight resistance genes (R genes) encode nucleotide-binding proteins which serve as specific receptors for effector proteins of the fungal pathogen which are deposited in the host cytoplasm (HAVERKORT et al. 2016).

Resistance to apple scab disease (which is caused by the ascomycete fungus *Venturia inaequalis*) in apple trees was induced by inserting an endogenous apple scab resistance gene *hcrVf2* under the control of its own regulatory sequences by *Agrobacterium*-mediated transformation technique (VANBLAERE et al. 2011, VANBLAERE et al. 2014). Following chemical activation of the recombination mechanism resistant cisgenic plants were selected from the initial transformants (VANBLAERE et al. 2011, VANBLAERE et al. 2014). The scab resistance gene *hcrVf2* is one of several resistance genes of the Vf locus of *Malus floribunda*, although only two of these genes are involved in resistance to apple scab (MALNOY et al. 2008). The function of the Vf genes in conferring resistance to the fungal disease is still to be elucidated, but is supposed to be based on the interaction between the resistance inducing gene products and specific pathogen factors (MALNOY et al. 2008, MALNOY et al. 2016).

Grape plants displaying resistance to the fungi *Botrytis cinerea* and *Erysiphe necator* were generated by transgenic modification with endochitinase genes derived from another fungus (e.g. *Trichoderma* spp.) and grafting onto non-transgenic rootstocks for field trials (RUBIO et al. 2015). The genes play an important role in the control of fungal pathogens, e.g. by exhibiting chitinolytic functions.

Similarly, higher tolerance to the fungal pathogen *Phytophthora citrophthora* in orange trees was achieved by creating a transgenic orange overexpressing a pathogenesis-related protein, the PR P23 osmotin-like protein (FAGOAGA et al. 2001). These PRs are encoded by

the host plant and expression is induced systemically in the infected plants. *In vitro*, the P23 protein inhibits the growth of several phytopathogenic fungi and oomycetes (FAGOAGA et al. 2001). In the EU field trials have been conducted with transgenic rootstocks of orange and non-transgenic clementine scions.

Table 4: Overview of plants with disease-resistance traits developed by nGMs

DR trait class	Plant	nGM used	Molecular mechanism	Literature
Virus resistance	*Arabidopsis thaliana*	SDN-1 CRISPR/Cas 9	Point mutation of a viral host factor (eIF4E)	PYOTT et al. (2016)
	Cucumber	SDN-1 CRISPR/Cas 9	Deletions and single nucleotide polymorphisms (SNPs) of eIF4E	CHANDRASEKARAN et al. (2016)
	Potato	Intragenesis	Site-directed mutagenesis of R-gene and modified eIF4F	CAVATORTA et al. (2011), DUAN et al. (2012)
	Tobacco	Transgrafting	RNAi silencing of CMV genes	Bai (BAI et al. 2016)
	Tomato	Transgrafting	Post-transcriptional gene silencing	LEIBMAN et al. (2015)
	Lime	Transgrafting	RNAi against viral genes	Soler et al. 2015
	Plum	Transgrafting	RNAi silencing of viral CP genes	SIDOROVA et al. (2016)
	Plum	Transgrafting	Post-transcriptional gene silencing of viral RNA	POLÁK et al. (2017)
	Grapefruit, citrus	SDN-1 CRISPR/Cas9	Modification of canker susceptibility gene (CsLOB1)	JIA et al. (2017)
Bacterial resistance	Rice	CRISPR/Cas9, TALEN	Disruption of susceptibility gene (OsSWEET13)	LI et al. (2012), ZHOU et al. (2015)
	Grapevine	Transgrafting	Expression of transgenic antimicrobial gene	LI et al. (2015)
	Citrus	Transgrafting	Overexpression of modified plant thionin	HAO et al. (2016)
	Walnut	Transgrafting	Expression of gene mediating post-transcriptional gene silencing of bacterial genes	OECD (2016), ESCOBAR et al. (2002)
	Tomato	SDN-1 CRISPR/Cas9	Loss-of-function mutation in susceptibility gene	NEKRASOV et al. (2017)
Fungal resistance	Wheat	TALEN	Mutation of susceptibility genes	WANG et al. (2014a)
	Grapevine	RNA	Mutation (knock-down)	PESSINA (2016)

		interference	of *mlo* susceptibility gene	
	Apple	Cisgenesis	Expression of resistance gene	VANBLAERE et al. (2011), VANBLAERE et al. (2014)
	Potato	Cisgenesis	Expression of resistance genes	HAVERKORT et al. (2016)
	Grapevine	Transgrafting	Expression of endochitinase genes	RUBIO et al. (2015)
	Orange	Transgrafting	Overexpression of fungal resistance protein	OECD (2016), field release EU

Plants with virus resistance, fungal resistance as well as bacterial resistance are also developed by conventional breeding approaches and classic GM technology (see Table 2). COLLINGE et al. (2008) provide an overview of potential strategies to create GM disease resistant plants and provide examples. Three major strategies to achieve disease resistance are outlined by the authors:

- Direct interference with the pathogen or its physiology, e.g. by adding genes that encode antimicrobial proteins.
- Regulation of the natural induced host defences, e.g. recognition of the pathogen (by adding resistance genes) or exploiting regulatory pathways (e.g. signal transduction).
- Pathogen mimicry or "genetic vaccination" e.g. by gene silencing (Collinge et al. 2008).

A recent overview on GM applications with resistance against biotic stress factors, including DR, is provided in Kohl (2018). Risk issues which were discussed for applications of GM DR plants are highly relevant for the discussion of potential adverse effects which may be associated with nGM DR plants.

Risk issues relevant for nGM plants with DR traits

Some approaches to achieve virus resistance are based on RNA-mediated gene silencing which can be subsumed as a pathogen mimicry strategy (COLLINGE et al. 2008).

Environmental risks addressed in the ERA of virus resistant GMPs comprise increased persistence, weediness and invasiveness of the GM plant or wild relatives which acquired virus resistance, as well as impacts on non-target organisms (US APHIS 2006).

The risk assessment of GM plants for RNAi-mediated pest control has been addressed by several authors (e.g. (CASACUBERTA et al. 2015, ROBERTS et al. 2015). RAMON et al. (2014) discussed relevant aspects for the food and feed and environmental risk assessment of RNAi-GM plants. For the food and feed risk assessment the current approach was considered appropriate, although the focus should be on the RNAi molecules rather than on novel (GM) proteins (RAMON et al. 2014). Similar issues are discussed by CASACUBERTA et al. (2015) in relation to human health and the environment. Due to the lack of novel proteins expressed in the GMP, toxicological or allergenic effects are considered less relevant for RNAi-based plants expressing only RNAs and no novel proteins (CASACUBERTA et al. 2015).

However, other health and environmental risks may be regarded important, such as off-target gene silencing.

Some nGM approaches use GM techniques to introduce resistance traits into a plant in combination with transgrafting, i.e. grafting of non-GM scions on GM rootstocks. Expression of fungal or antimicrobial genes resulting in novel proteins as well as gene-silencing via RNAi without detectable proteins have been used in such applications. Risks of newly expressed proteins to the environment and human health will be similar to GM approaches if these are transported from the GM rootstock to the non-GM scion and will depend on the type and abundance of the protein (COGEM 2009). Hence, for newly expressed gene products such as plant antimicrobial genes or fungal endochitinase genes, risks will have to be assessed on a case-by-case basis. DR based on gene silencing by RNA interference and subsequent transgrafting are subject to similar concerns and potential adverse effects as for GM approaches. If novel proteins are not passed on to the scion, environmental risks may be restricted to the GM rootstock and its environmental exposure, in particular to soil ecosystems. For post-transcriptional gene silencing in combination with transgrafting similarly the transmission of the RNAi to the scion will determine potential risks, e.g. the role of the target gene in plant physiology and potential off-target effects (CASACUBERTA et al. 2015). Similarly, human health risks depend on the likelihood of transmission of RNAi-signals, proteins or metabolites from the GM rootstock to the non-GM signals, as products from the non-GM scion are mostly used for consumption (COGEM 2009). Potential adverse effects may be due to the mobility and type of novel product expressed in the GM rootstock (SCHAART&VISSER 2009, SONG et al. 2015).

Other aspects have to be considered for applications to induce virus resistance by transgrafting. LEMGO et al. (2013) identified several concerns that should be addressed during risk assessment. These include pleiotropic silencing effects, effects of the transgenic rootstock on non-target (soil) organisms, gene transfer of virus resistance to wild type plants resulting in increased fitness and invasiveness, potential development of novel viral strains and food safety aspects.

Potential environmental impacts which were discussed for plants with resistance to bacterial pathogens comprise effects on plant-associated bacterial communities, in particular in the rhizosphere (HEUER et al. 2002, LOTTMANN et al. 2010). Concerns for antimicrobial proteins expressed in GM plants refer to potentially allergenic or toxic properties for vertebrates or humans (COLLINGE et al. 2008). SCHLAICH et al. (2006) indicated that novel proteins such as Kp4 expressed in GM plants may raise biosafety issues for human health. Also effects on bacteria, such as resistance to antibiotics were mentioned for these types of disease resistant GMPs (COLLINGE et al. 2008).

Environmental and health implications of cisgenic/intragenic GM potatoes with resistance to fungal pathogens were evaluated by US APHIS in the context of the petitions for deregulation of W8, X17 and Y9 potatoes with late blight resistance (e.g. Aphis petition No. 16-064-01p). The expressed VNT1 protein (an R protein) does not directly target a pest or act as a toxin but activates a response within the host plant upon pathogen contact. The proteins are expressed at low levels in the GMP, and a range of homologs are present in potato. US APHIS concluded that the VNT1 protein does not have amino acid sequence similarities with known allergens or protein toxins and would not have adverse effects to human or animal health. Environmental concerns for fungal resistant GMPs comprise

potential adverse effects on chitin-containing non-target organisms, e.g. in the case of chitinase-expressing GMPs. No effects on fungal root symbionts were observed in chitinase-expressing GMPs (VIERHEILIG et al. cited in BRUINSMA et al. (2003). However, negative effects of GM-expressed chitinase on symbiotic mycorrhizal fungi were suggested to be a potential cause for the lack of resistance to certain fungal diseases in field trials with GM silver birches (PASONEN et al. 2004, PASONEN et al. 2005). For fungal resistant GMPs which express resistance genes (R genes, e.g. the *vnt* gene), the potential adaptation of the targeted pathogens with specific avirulence genes corresponding to the specific R gene has been mentioned as a potential concern (COLLINGE et al. 2008).

Applications of genome editing using site-directed nucleases (e.g. CRISPR, TALEN) may be accompanied by risks due to changes in the targeted host-pathogen relationship. Disruption of the functionality of R genes or S genes may trigger mutations in the pathogen restoring its ability to successfully infect and spread in the host.

Also the induction of pleiotropic phenotypes in the plant may be an unintended consequence. Resistance to powdery mildew, a fungal disease, was established by knocking out plant susceptibility (*mlo*) genes by genome editing. However, a number of pleiotropic effects such as reduced plant size or premature senescence were described (KUSCH&PANSTRUGA 2017) most likely because the knocked out plant genes may have several other functions as well. Also knock-out or silencing of members of the *mlo* gene family that are not involved in pathogen susceptibility by off-target activity may lead to unintended effects on physiology, development or composition with implications for food, feed and environmental safety (PESSINA 2016).

5.2.3 Environmental effects associated with nGM plants with compositional changes

A wide range of plants with changed composition have been developed in recent years by nGM approaches, exploiting the available knowledge on metabolic pathways in plant cells and the ability to specifically target and modify particular enzymatic functions within these metabolic networks. nGM methods are used as tools for basic research to elucidate biosynthetic pathways as well as to engineer plants with changed composition for future commercialization as food or feed products or as industrial feedstocks. An overview on recent developments which are relevant for the latter purposes is presented in the following.

nGM applications to modify plant composition

nGM plants with altered composition were developed in a broad range of plant species, including important staple crops such as rice, wheat, maize, soybeans and potato. In addition other oilseed plants e.g. *Camelina*, as well as fruit tree species, such as apple, and edible mushrooms were modified for content.

The list of examples for the targeted traits includes traits to alter the content of nutritionally important metabolites, e.g. either to increase the content of desired substances, such as specific lipids and anthocyanin or to decrease the content of unwanted components such as acrylamide precursors or gluten proteins among others. Other content traits are targeted to improve industrial use of the respective feedstocks, e.g. by modification of lipid composition of oilseed plants, modification of starch content in potato or the reduction of lignin in sugarcane. A third set of modifications is introduced to improve quality traits which influence

the storage and processing of products, e.g. to preserve the compositional quality of rice upon storage or to decrease the browning of sliced mushrooms.

These modifications were mostly developed by genome editing approaches, particularly by SDN-1 applications aimed to knock out the expression of endogenous plant genes with a specific biosynthetic function. However, some applications were also developed by other nGM methods such as cisgenesis/intragenesis and RdDM/transgrafting. An overview of applications retrieved from the scientific literature with a potential for future commercialization is given in Table 5.

Table 5: Overview of plants with altered composition developed by nGMs

nGM approach	**Plant**	**Specific type of nGM used**	**Molecular mechanism**	**Literature**
Genome Editing (by SDNs)	*Camelina sativa*	SDN-1 CRISPR/Cas9	Knock-out of three homoeologous FAD2 genes to enhance fatty acid composition in seed (increase of oleic acid content to 50%)	JIANG et al. (2017), MORINEAU et al. (2017)
	Potato	SDN-1 CRISPR/Cas9	Multiallelic knock-out of granule-bound starch synthase (GBSS) gene to alter starch quality	ANDERSSON et al. (2017)
	Potato	SDN-1 TALEN	Knock-out of the vacuolar invertase gene to improve cold storage and processing	CLASEN et al. (2016)
	Rice	SDN-1 CRISPR/Cas9	Knock-out of starch branching enzyme genes (SBEI and SBEIIb) to increase amylose content	SUN et al. (2017a)
	Rice	SDN-1 TALEN	Knock-out of lipoxygenase LOX3 to decrease dioxygenation of polyunsaturated fatty acids in stored rice seeds	MA et al. (2015)
	Rice	SDN-1 TALEN	Simultaneous knock-out of three genes (OsBADH2, OsCKX2 and OsDEP1) to produce fragrant rice with increased 2-acetyl-1-pyrroline content	SHAN et al. (2015)
	Mushrooms	SDN-1 CRISPR/Cas9	Knock-out of endogenous genes to suppress browning of wounded tissue	See: WALTZ (2016a)
	Soybean	SDN-1 CRISPR/Cas9, CRISPR/Cpf1	Knock-out of the FAD2-1A and FAD2-1B genes to increase levels of oleic acid and lower content of polyunsaturated fats	HAUN et al. (2014), KIM et al. (2017a)
	Soybean	SDN-1 TALEN	Knock-out of the FAD3 genes in a fad2-1a and fad2-1b soybean line to increase linolenic acid	DEMOREST et al. (2016),
	Sugarcane	SDN-1 CRISPR/Cas9	Knock-out of the caffeic acid O-methyltransferase to decrease lignin content	JUNG&ALTPETER (2016)
	Maize	SDN-1 ZFN	Knock-out of phytase genes to reduce phytate content	SHUKLA et al. (2009)

	Maize	SDN-1 TALEN	Knock-out of ZmPDS, ZmIPK1A, ZmIPK, ZmMRP4 to modify phytic acid biosynthesis and phosphorus storage	LIANG et al. (2014)
	Wheat	SDN-1 CRISPR/Cas9	Knock-out of different α-gliadins to reduce content of anti-nutritional gluten	SANCHEZ-LEON et al. (2018)
	Tobacco	SDN-1 TALEN	Knock-out of two (1,3)-fucosyltransferase and the two (1,2)-xylosyltransferase genes for production of specifically glycosylated antibodies	LI et al. (2016b)
Intragenesis	Potato	-	Silencing of asparagine synthetase-1 (StAst1) to reduce acrylamide-forming potential in tubers	CHAWLA et al. (2012)
	Grape vine	-	Increased anthocyanin content	See: VOGEL (2016)
Cisgenesis	Apple	-	Increased anthocyanin content	See: SCHAART et al. (2016)
RdDM, Transgrafting	Potato	Introduction of RNA expression construct by transgrafting	Silencing of GBSSI to reduce amylose starch content	KASAI et al. (2016b)

The traits are either developed by modification of single target genes (including multialleleic knock-out or downregulation of expression) or they are due to complex modifications, i.e. changes to a number of different genes involved in the targeted biosynthetic pathways, e.g. knockout of 35 different alpha-gliadin genes in wheat using a multiplexed approach (SANCHEZ-LEON et al. 2018). These genes can be related, i.e. as members of a gene family or the different targeted genes can be functionally unrelated and their modification will influence different steps in the targeted biosynthetic pathways.

As can be seen in Table 5 the targeted traits on the one hand affect key cellular metabolic pathways, such as sugar and lipid biosynthesis, or they modify production of secondary metabolites in the respective plant species.

A number of comparable traits to modify the composition of plants were also developed by GM technology. A recent overview of these applications is provided in KOHL (2018). GM applications modify plant composition by inserting transgenic constructs to either express heterologous biosynthetic genes from related or unrelated (plant) species or to produce transgenic RNAs to downregulate or silence the expression of endogenous genes. A number of these GM plants were field tested in the EU or other countries, or authorized for commercial use in non-EU countries. Some GM soybean lines were authorized in the EU since 2015 for import and food and feed use: MON 87705 and 305423 with an increased oleic acid and reduced linoleic acid content, MON 87769 with increased omega-3 fatty acid content and two double stacked events of MON 87705 and 305423 with herbicide resistant GM soybeans[9].

[9] https://ec.europa.eu/food/plant/gmo/eu_register_en

Risk issues relevant for nGM plants with changed composition

Based on experience with problem formulation for the risk assessment of GM plants (EFSA 2010, EFSA 2011) a number of potential risk issues regarding food and feed safety and environmental effects should be addressed in the risk assessment of nGM plants from this class, particularly any toxic or allergenic effect resulting from proteins with modified sequence, or any anti-nutritive effect of newly produced metabolic compounds.

An important issue for risk assessment of nGM and GM plants with altered composition is that the resulting organisms are “substantially different” from the parental plants or existing crops. In consequence a history of safe use as food and feed products will be obsolete for a number of such plants. As exemplified by several products, the changes may be complex and affect a number of different compounds. If key metabolic function are modified, some compositional changes may be unintended, e.g. due to pleiotropic effects of the introduced modifications.

The nutritional effects of complex compositional changes may be difficult to predict and should be subject to a thorough assessment of food and feed safety. This risk assessment should take into account that health effects may depend on the level of exposure, i.e. the amount of these products included in different diets. Furthermore the different sensitivities of population sub-groups (e.g. children, people with sensitivities to food components or allergies, people with physiological disorders) to the respective components need to be considered during such an assessment. As health consequences are not fully understood and respective data may not be available, specific assessment of such nGM products is justified.

Compositional changes can furthermore result in environmental effects, e.g. due to altered interactions with herbivorous animals, e.g. for nGM plants with increased sugar or modified lipid contents. Respective effects are discussed for GM oilseed plants producing two bioactive omega-3 long-chain fatty acids, which are involved in key physiological functions in invertebrates and vertebrates (COLOMBO et al. 2018). These fatty acids are known to be synthesized by primary producers in aquatic ecosystems, but not by terrestrial plants. Therefore, cultivating modified GM oilseed crops represents a shift in the accessibility of such fatty acids to terrestrial consumers and may have potential ecological and evolutionary consequences, e.g. by changing the physiology and survival of wildlife such as herbivores (COLOMBO et al. 2018).

Furthermore plants with changes in composition might have an increased attractiveness to plant pests or pathogens. Compositional changes may also have effects on phenotypic, e.g. morphological, characteristics, such as stability, e.g. for nGM plants with reduced lignin content. Morphological changes were reported for GM soybeans which were modified to silence omega-3 fatty acid desaturase (SINGH et al. 2010). The plants displayed significantly larger and heavier seeds and exhibited enhanced susceptibility to virulent *Pseudomonas syringae* as well as bean pod mottle virus (SINGH et al. 2010).

5.2.4 nGM applications with enhanced fitness against environmental stressors or alteration of morphological or reproductive characteristics

A variety of different traits with environmental/ecological relevance that have been established by nGM approaches such as genome editing and transgrafting are reported in

the literature. Such applications were developed in model plants for research, e.g. Arabidopsis, as well as in (crop) plants of agricultural importance, e.g. oilseed rape, rice and tomato.

Most of these applications have been developed by genome editing via SDN-1 mediated knock-out of endogenous plant functions. Some of the traits were established by transgrafting. An overview of the respective applications is presented in Table 6.

Table 6: Overview of plants developed by nGMs with enhanced fitness or altered morphological or reproductive characteristic

nGM approach	**Plant**	**Specific type of nGM used**	**Molecular mechanism**	**Literature**
Genome editing (by SDN)	*Arabidopsis thaliana*	SDN-1 CRISPR/ Cas9	Use of CRISPR/Cas9 with truncated gRNA to generate new alleles of OST2 to alter stomatal closing responses for increased environmental stress resistance.	(OSAKABE et al. 2010), (WATANABE et al. 2016)
	Arabidopsis thaliana	SDN-1 CRISPR/ Cas9	Knock-out of CBF1 and CBF3 genes increases expression of CBF2 and induces cold and freeze tolerance	(Zhao CZ, Zhang et al. 2016, Zhao and Zhu, 2016)
	Tomato	SDN-1 CRISPR/ Cas9	Knock-out of SlAGL6 to enable facultative parthenocarpy and fruit production under heat stress.	(KLAP et al. 2017)
	Tomato	SDN-1 CRISPR/ Cas9	Knock-out of the florigen paralog and flowering repressor SP5G to decrease day-length sensitivity of flowering in tomato.	(SOYK et al. 2017)
	Tomato	SDN-1 CRISPR/ Cas9	Knock-out of the SlCLV3 promoter in tomato to generate larger fruit and increased numbers of flower buds	(RODRÍGUEZ-LEAL et al. 2017)
	Oilseed rape	SDN-1 CRISPR/ Cas9	Knock-out of two ALCATRAZ (ALC) homoeologs to induce increased resistance of seed shattering from mature fruits	(BRAATZ et al. 2017)
	Rice	SDN-1 TALEN	Modification of several endogenous genes: (OsCSA, OsPMS3, OsDERF1, OsGN1a, OsTAD1, OsMST7 and OsMST8) to generate relevant agronomic phenotypes (e.g. photoperiod-sensitive male sterility, enhanced grain yield, drought resistance, etc.)	(ZHANG et al. (2016b)
	Rice	SDN-1 CRISPR/ Cas9	Knock-out of GW2, GW5 and TGW6 to increase grain weight.	(XU et al. 2016b)

	Rice	SDN-1 CRISPR/ Cas9	Editing of early heading date genes (HD 2, 4, 5) to generate early-maturing rice cultivars.	(LI et al. 2017e)
	Rice	SDN-1 CRISPR/ Cas9	Editing of four yield-related genes Gn1a, DEP1, GS3, and IPA1 in Rice genes to create mutants showing enhanced grain number, dense erect panicles, and larger grain size (LI et al. 2016c)	(LI et al. 2016c)
	Maize	SDN-3 CRISPR/ Cas9	SDN-3 mediated insertion of a heterologous promoter to create overexpressing variants of ARGO58 to improve grain yield under drought conditions (reduced ethylene sensitivity).	(SHI et al. 2017a)
Trans-grafting	Rootstock: GM apple; Scion: non-GM apple	-	Overexpression of a peach PpCBF1 gene in apple to generate enhanced freezing tolerance, instead resulting in reduced growth and delayed flowering.	(ARTLIP et al. 2016)
Trans-grafting	Rootstock: GM bottle gourd; Scion: non-GM watermelon	-	GM rootstock expressing the Arabidopsis CBF3/DREB1A gene to induce a cold-response pathway leading to increased cold tolerance	(CHO et al. 2017)
Trans-graftinig	Rootstock: GM bottle gourd; Scion: non-GM watermelon	-	Bottle gourd expressing the Arabidopsis H+-pyrophosphatase AVP1 gene to generate a transgenic bottle gourd as a salt-tolerant rootstock for improved watermelon production under non-favourable conditions.	(HAN et al. 2015b)
Trans-grafting	Rootstock: GM cherry colt; Scion: non-GM sweet cherry	-	GM cherry colt as a rootstock to generate dwarfed cherry trees with reduced canopy size and longer leaf retention in autumn for non-GM sweet cherries.	(RUGINI et al. 2015)
Trans-grafting	Rootstock: GM tomato; Scion: non-GM tomato	-	Two constructs with inverted repeat RNAs from fatty acid desaturase gene (LeFAD7) expressed in a transgenic rootstock for gene silencing to induce high temperature tolerance in the non-GM scion	(NAKAMURA et al. 2016)

A significant number of the applications generated by genome editing were developed by modification of multiple genomic target genes, e.g. three genes in Arabidopsis that directly regulate cold responsive (SHI et al. 2017b) (ZHAO et al. 2016), flowering suppressor genes that negatively control the heading date of rice varieties (LI et al. 2017f) and genes involved in regulation of seed shattering of mature fruits in oilseed rape (BRAATZ et al. 2017).

An application in maize demonstrates that SDN-3 approaches may also be used to develop traits to increase resistance against environmental stress factors (SHI et al. 2017a).

Many of the applications need to be evaluated by further research for their potential for commercial development. However, further development of some of the applications for commercialization may be pursued, namely for applications to develop traits of agronomic importance in relevant crops, e.g. traits in rice to increase yield (LI et al. 2016c) (Shi, J.R. et al 2017) (ZHANG et al. (2016b) (XU et al. 2016b).

Two recent publications (LI et al. 2018, ZSÖGÖN et al. 2018) indicate the potential of genome editing for an approach called *de novo* domestication, i.e. to rapidly develop crop lines from wild forms with desired properties like strong resistance towards pathogens or salt tolerance. In both cases characteristics associated with domesticated tomato plants were established in different lines of *Solanum pimpinellifolium* by simultaneously editing only 4 or 6 genomic loci, respectively, while maintaining the desired resistances present in the wild lines. Among the introduced domestic characteristics were increased fruit number, size, shape and nutrient content of fruits as well as plant architecture and growth characteristics. The authors regard their approach as a viable route for the direct development of new crop varieties from wild plants in order to exploit their genetic diversity and thus as a fast and simple alternative to classic breeding programs.

As illustrated in KOHL (2018) also GM technology is used in various ways to develop GM plants (e.g. maize, barley, rice, soybean, wheat, plum) with resistance to abiotic stress (KOHL et al. 2018, Table 3) and GM plants (e.g. oilseed rape, false flax, wheat, apple) with relevant agronomic properties (KOHL et al. 2018, Table 5). However, most of these applications are currently still field tested (mostly in non-EU countries) and not ready for marketing yet.

Risk issues relevant for nGM plants with enhanced fitness or altered morphological or reproductive characteristics

Traits related to enhanced fitness or altered phenotypical properties may trigger a variety of (ecological) effects which are considered for adversity in the current risk assessment framework for GM plants that was developed by EFSA (EFSA 2010, EFSA 2011). As indicated above most of the applications are based on multiple modifications resulting in complex changes and they have to be evaluated in relation to a complex environment. Against a background of insufficient knowledge on the cellular mechanisms underlying the respective traits and on interaction of the modified plants with their environment the general safety of such applications may not be assumed with confidence. The situation rather gives reason to require a risk assessment for such nGM plants according to the principles and guidance established for comparable GM plants.

Adverse effects may be due to an increased potential for spread, invasiveness or weediness of the modified plants. However, respective adverse effects may also result from hybridization of the respective nGM plants with sexually compatible species and introgression of such traits into related species. However, depending on the modified trait and the wild relative(s), effects of such outcrossing events can be adverse for different reasons: in the case of related valued native species a decrease in reproduction or fitness would be regarded as adverse, similar as an increase of reproductive fitness in case of the weedy relatives.

Applications that target morphology, such as plant architecture or yield parameters, or reproductive characteristics, e.g. flowering time, may be associated with impacts on different wild living animal species upon cultivation. Affected species may be herbivores associated with the respective plants or pollinating species in the latter case. Further effects on the exposed agricultural ecosystems or neighboring habitats and their biodiversity may result from such initial impacts.

The risk assessment should also consider unintended effects, e.g. due to changed composition as a secondary effect of the modifications. Such effects could plausibly be associated with applications to increase yield and fruit/seed production. However, also with other nGM applications such secondary effects were found: FREIMAN et al. (2015) report that a transgrafting experiment with apple showed that the resulting phenotype of delayed senescence was accompanied by an elevated chlorophyll content in the leaves. In another example an application of genome editing aimed at base editing of a nitrogen transporter gene involved in nitrogen metabolism resulted in a significantly semi dwarf phenotype in rice plants (LU&ZHU 2017).

Furthermore applications of *de novo* domestication, e.g. as reported recently for wild tomato (LI et al. 2018, ZSÖGÖN et al. 2018), will also result in nGM plants which may differ in a significant number of (compositional) characteristics from domesticated tomato lines which are commonly used for food production. Unlike the latter lines *de novo* domesticated plants also do not have a history of safe use, and thus should undergo a premarket risk assessment.

5.3 Risk assessment of unintended effects associated with nGMs and nGM plants

5.3.1 Types and sources of unintended changes

As indicated in chapter 5.1.3, the presently available nGMs, are not sufficiently specific to introduce just the intended molecular changes into plants. Thus additional unintended molecular changes may be introduced by a particular nGM method and they may lead to phenotypic effects affecting the properties of the modified plant (SAM 2017).

In general several types of unintended effects can be distinguished (AGAPITO-TENFEN et al. 2018):

- Unintended changes at genomic locations other than the genomic target site(s) for intended modifications; i.e. modifications which are usually not genetically linked to the desired trait(s)
- Unintended molecular changes in the vicinity of the intended site of modification; i.e. changes different from the intended modifications, but tightly linked to the desired trait(s)
- Unintended effects different to the desired trait(s) that are due to the modifications at the genomic target; i.e. pleiotropic effects of the intended modification(s) linked to the desired trait(s)

Unintended changes may modify the expression of endogenous genes and impact the plant's metabolism and phenotype. According to the nature of these particular phenotypic

effects, the unintended changes may be considered either harmless or adverse in terms of human health and the environment.

The existing evidence to assess the possibility and the effects of method-related changes that may be associated with different nGM approaches is limited at present. This is partly due to the limited experience with some newly developed nGMs. It is also due to the fact that the current focus of research is more on method development and the exploration of possible applications of the different nGMs than on biosafety issues. However, there is evidence indicating that the above mentioned issues should not be disregarded without a proper assessment.

Method-related unintended molecular changes may be associated with different aspects of the overall development process of nGM products. They depend either on the mechanisms of the particular nGM or on the characteristics of further methods required for the overall development of a particular nGM plant, such as methods for *in vitro* cultivation of plant cells and tissues, methods to facilitate the uptake of nGM components (e.g. protoplast transfection methods) or methods for the regeneration of plants from cultivated cells or tissues.

5.3.2 **Unintended changes due to transformation and transfection methods**

Typically exogenous effector molecules need to be introduced into recipient plant cells to initiate nGM processes, such as (i) recombinant DNA constructs for stable genetic transformation of plant cells, e.g. to express nucleases for genome editing or other molecular tools required for a particular nGM; (ii) recombinant DNA constructs for transient expression of nGM-related components (RNA or proteins required for the respective nGMs); (iii) specific DNA, RNA or ribonucleoprotein complexes. Unintended genetic or epigenetic changes can be introduced as a side effect of transformation or the transfer of method-related components into the recipient cells (LATHAM et al. 2006, MEHROTRA&GOYAL 2012).

Unintended changes may also result from the integration of genetic constructs into the recipient genome of plant cells for nGM approaches that involve the use of GM techniques. This relates to e.g. cisgenesis/intragenesis, the transformation of rootstocks for transgrafting and genome editing approaches that are based on the expression of SDN components from transgenic constructs. It is typically a random process and thus can result in unintended genetic changes, e.g. by the disruption of functionally important genomic sequences or due to the integration of other unrelated DNA sequences (SAM 2017). Untargeted integration of non-endogenous sequences can also modify the expression of endogenous genes located in the vicinity of the integration site(s) (LADICS et al. 2015).

It should also be noted that genetic constructs that are only transiently introduced into plant cells to express method-related components may integrate into the genome of the recipient cells. If transgenic constructs should only be present during intermediate steps it is important to assess whether all such modifications are indeed fully removed and absent from the final product. This relates to any inserts of the constructs for expression of method components as well as to secondary inserts, e.g. of vector backbone sequences. BRAATZ et al. (2017) for example found by way of whole genome sequencing (WGS) that transformation of oilseed rape with a CRISPR/Cas9 expression construct resulted in at least five independent insertions of vector backbone sequences in the genome of the modified plant.

5.3.3 **Unintended changes due to off-target activity**

Unintended genetic and epigenetic changes may also result from the respective particular nGM mechanism. Well known examples are off-target modifications associated with genome editing. They typically happen in genomic sequences that share a sufficiently high degree of similarity with the target loci and thus can associate with the molecular editing tools leading to off-target edits (KANCHISWAMY et al. 2016, YEE 2016). Off-target activity can also be associated with other nGM, e.g. RdDM approaches. In such cases not only the target site(s) are epigenetically modified, but other genomic locations as well (GALONSKA et al. 2018).

The frequency of off-target effects as well as their extent and distribution in the genome are different for the various genome editing approaches and depend on both the targeting characteristics of the particular approach and on the specific method used for genome editing (HCB 2017, WOLT 2017), including the exact experimental protocol (YEE 2016).

According to YEE (2016) off-target activity depends on

- the frequency of homologous sequences in the genome
- the characteristics of the specific nuclease type
- the expression level of the nuclease
- the time span for which the nuclease is present in the target cell
- the accessibility of the homologous sequence and of any potential off-target sequences in the chromatin

The accessibility of DNA genomic regions to some nucleases used in genome editing, especially to MNs, ZFNs and TALENs, depends e.g. on their specific methylation pattern (GUHA et al. 2017). Other factors influencing off-target activity are explained in the following.

CRISPR nuclease variants with enhanced specificity were developed to reduce off-target activity, such as a modified, high-fidelity Cas9 or nucleases from other bacteria with an intrinsically higher specificity, e.g. Cpf1 (KLEINSTIVER et al. 2016, ZHAO&WOLT 2017). Unwanted off-target activity could be reduced through transient expression of nuclease components and by expression at reduced levels and in specific cell types or developmental stages (YEE 2016). Also various other methods are developed to limit the activity of SDNs in target cells, including the use of inducer or repressor molecules to control the expression or activity of the respective nucleases (PAWLUK et al. 2016). Furthermore, fewer off-target changes occurred when functional nuclease molecules were preassembled and directly introduced into recipient cells, instead of delivering SDN components as genetic constructs (GUHA et al. 2017, HILSCHER et al. 2017, LIANG et al. 2017b).

Different approaches may be used to limit the off-target activity of SDN-mediated genome editing. First developers can select and apply suitable methods with a high level of specificity taking into account the above mentioned factors. Furthermore off-target activity can also be influenced by the choice of the specific genomic target sequence, e.g. by selecting target sequences which display a low homology to other genomic sequences, in order to limit the number of unintended binding sites throughout the respective plant genome.

A number of different approaches to predict and identify off-target activity is available for use in risk assessment as outlined e.g. in AGAPITO-TENFEN et al. (2018).

Bioinformatic tools and special software help to predict genomic target sites and design suitable SDNs (described in (KANCHISWAMY et al. 2016, ZHAO&WOLT 2017). There are concerns, however, that such *in silico* screening/identification for off-target sites may not reliably identify all *in vivo* off-target sites. Thus for genome editing of animal cells new approaches to assess off target activity have been suggested, see e.g. AKCAKAYA et al. (2018), which may be also employed for genome editing to modify plants. In addition calls have been issued to also consider and investigate potential target sites with lower cutting probabilities (CHAKRABORTY 2018).

A suite of *in vitro* and *in vivo* methods is available to identify sites of potential off-target activity in the genome; some of them, including Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing (GUIDE-seq), High-Throughput Genomic Translocation Sequencing (HTGTS), Breaks Labeling, Enrichments on Streptavidin and Next-Generation Sequencing (BLESS), and Digested Genome Sequencing (Digenome-seq), can provide unbiased whole genome screens for such sites (KANCHISWAMY et al. 2016, ZISCHEWSKI et al. 2017). Additionally the final genome edited plants can be checked with WGS and biochemical methods for potential off-target modifications (ZISCHEWSKI et al. 2017). However, testing by WGS may be constrained by technical limitations, e.g. if sequence information from repetitive sequences cannot be obtained (SAM 2017). If adequate reference genomes are not available additional efforts to generate whole genome data from the parental line are required to conduct the comparison to identify unintended sequence changes.

In recent years a number of genome editing applications in plants were checked for off-target changes. HILSCHER et al. (2017) concluded that overall levels of untargeted mutational changes throughout the plant genome were not elevated. However, their review included several reports that identified off-target edits at genomic locations which were very similar to the target sequence (see (HILSCHER et al. 2017). Another report noted unexpectedly high off-target activity (ZHANG et al. 2016e). Furthermore recent research has shown that assumptions regarding the level of specificity associated with a particular SDN may not always hold true. In a specific case a modified Cas9 nuclease with less stringent requirements for matching a specific protospacer adjacent motive (PAM) unexpectedly displayed a higher overall specificity (HU et al. 2018). Recent reports from genome editing experiments in mammalian cells indicate that significant numbers of larger deletions were caused by CRISPR/Cas9-mediated genome editing using different methods, including stable transformation with SDN expression constructs, transient expression of CRISPR/Cas and transfection with functional CRISPR ribonucleoprotein complexes (KOSICKI et al. 2018). In addition to genetic modifications at target sequences different kinds of secondary modifications (point mutations, indels, deletions and insertions) were found at distant genomic loci (KOSICKI et al. 2018). It needs to be seen whether these results are also relevant for plant systems. However, it illustrates that assumptions regarding the high degree of specificity of genome editing approaches may not hold true as a general rule. It also underlines that current knowledge concerning prediction and detection of off-target modifications associated with genome editing is still limited and needs to be improved (WOLT 2017).

Uncertainties that remain regarding the occurrence of unintended effects cannot sufficiently be addressed by a rational design of the methods for genome editing. Developers have rather to resort to empirical testing of the efficiency and specificity of different method variants approaches to select methods with a good ratio of on-target efficacy vs. off-target activity, e.g. as described by KLEINSTIVER et al. (2016). Similarly appropriate approaches for the molecular characterization of nGM plants should be implemented to identify unintended effects during risk assessment.

The results can then be addressed by a targeted phenotypical assessment to determine the significance of the unintended effects identified. From a risk assessment point of view it is relevant to assess whether the respective unintended molecular changes are leading to phenotypic changes of an adverse nature (SAM 2017). Off-target modifications, which result in readily detectable phenotypic changes, can be identified and possibly eliminated during downstream breeding when generating elite lines (ZHAO&WOLT 2017). However, not all induced phenotypic changes can be easily detected: Significant alterations of important agronomic parameters, such as yield, fitness, growth and reproduction may be detected quite readily. Subtle changes e.g. in composition are more difficult to detect, however they may impact the nutritional quality or may be associated with allergenic or toxic effects. Also, some unintended changes may be genetically tightly linked to the desired trait(s) while others are not. That does influence how easily they can be removed, if at all. The probability that unintended changes are indeed removed depends on the number of breeding steps involved to establish a final breeding product. While this is less of a concern with annual crop plants which are typically subjected to a sufficient number of breeding cycles, this constraint is relevant for plants like trees, which do not undergo the same number of breeding cycles for practical reasons, as well as for plants which are mostly propagated vegetatively. On the other hand nGMs like genome editing may be used for direct modification of elite lines to speed up breeding processes (OECD 2018). However, faster ways of plant breeding may negatively impact the ability to safely remove any unwanted unintended modifications. Thus, strategies to minimize off-target activity and to identify unintended modifications should be implemented for the use of genome editing approaches to produce modified plants (SAM 2017).

5.3.4 Unintended changes due to other biotechnological interventions required for nGM approaches

Most nGM approaches require the use of further techniques to cultivate cells or explanted tissues (embryogenic or somatic tissues used for callus transformation or plant cells treated to yield protoplasts to facilitate transfection of genetic material or other method-related components), and methods to regenerate modified plants from single cells. A number of the genome edited plants reported in BORTESI&FISCHER (2015) as well as SCHAEFFER&NAKATA (2016) involved protoplast transfection which was used to deliver the genetic constructs for the expression of SDN reagents. Plant protoplast technology is also involved in DNA-free methods for genome editing. For such approaches functional site-directed nucleases, mostly CRISPR ribonucleoproteins, are introduced into protoplasts to initiate editing (KIM et al. 2017a, MALNOY et al. 2016). These approaches are currently considered and promoted as alternative to genome editing applications involving the delivery of DNA (KANCHISWAMY 2016, RAN et al. 2017). However, it is known that techniques such as protoplast technology, *in vitro*

cultivation of cells and regeneration of plants from cells and tissues are associated with unintended genetic changes (BAIRU et al. 2011, FILIPECKI&MALEPSZY 2006, HCB 2017, LADICS et al. 2015). These techniques can induce somaclonal variation which adds to the range of random genetic changes introduced by nGMs. While somaclonal variation is not a specific feature of nGM approaches, but can also happen in conventional breeding involving cell and tissue cultivation steps, some nGM methods dependent on methods known to result in somaclonal variation. It should thus be ensured that such changes are eliminated during subsequent steps of the breeding process.

5.3.5 **Unintended effects due to pleiotropic effects**

Some types of genetic modification can also give rise to pleiotropic effects, i.e. unintended secondary phenotypes which are also determined by the modified gene(s) and which are expressed along with the desired trait (SAM 2017). Pleiotropic effects can occur with traits developed by all types of breeding approaches, including nGMs. Pleiotropic effects will be present in the final breeding products, since they are tied to the desired trait(s). An example are nGM plants which were modified to have increased disease resistance due to the inactivation of susceptibility genes, namely the *mlo* genes conferring broad-spectrum resistance against powdery mildew fungi (KUSCH&PANSTRUGA 2017). A range of pleiotropic effects was found to be associated with the inactivation of certain *mlo* genes, including yield decrease and increased susceptibility to other fungal pathogens as well as effects on mycorrhizal development in roots (BROWN&RANT 2013). Data gathered in the course of screening for unexpected effects during the development process of nGM plants can support the risk assessment of unintended pleiotropic effects conducted in accordance with guidance established by EFSA (EFSA 2010).

Unintended effects may also be based on modifications/alterations, in particular disruption, of endogenous genomic sequences in proximity to integration sites of DNA introduced to develop plants by certain nGMs. Applications of cisgenesis, intragenesis or SDN-3 applications may be associated with such effects, depending on the characteristics of the integration site. Due to the genomic proximity of the integrated genetic elements and the altered genomic sequences flanking these elements, such unintended modifications cannot be removed by segregation during further breeding steps. Provided that their functions are understood the molecular characterization of the genomic sequences altered during the integration can provide indications as to whether unintended effects may arise. It may even be possible to predict the phenotype that may result from the modification.

5.3.6 **Assessment of unintended effects**

For the purpose of a comprehensive risk assessment of nGM plants unintended effects associated with all technical interventions involved in the process to develop a specific nGM plant have to be considered. A particular focus should be on unintended effects that may be predicted based on the specific characteristics of certain nGMs, such as off-target effects associated with a particular approach for genome editing. This can be addressed through an appropriate molecular characterization of the nGM application taking into account all procedures that were used. Information from the molecular characterization can then be used to address the question of whether the identified molecular changes may be tied to potential effects at the phenotypic level that should be further assessed.

For a robust characterization of unintended effects in nGM plants we recommend that risk assessors apply a 10 step approach outlined as follows. Steps 4 to 6 are specific for genome editing applications; the other steps are relevant for all nGM applications. The outlined steps are based on considerations discussed in more detail throughout this study:

1. Consider the specific characteristics of the applied nGM approach, including method particulars and the targeted plant species, to check whether it is known for a potential to induce unintended changes. This should include but not be limited to off-target activity. In the case of genome editing applications consider if the particular method has been optimised for precision, i.e. to result in a low level of off-target activity.
2. Check, if information is available from previous use of comparable approaches which is indicating a certain potential for unintended changes/off-target activity.
3. Assess the probability that genetically unlinked unintended modifications will be removed by crossbreeding used to develop a final product. This assessment should be based on the breeding history of the final product.
4. Use robust bioinformatics tools to predict potential sites for off-target changes in the reference genome of the respective plant species, if available. In case no adequate (reference) genome sequence data is available, use a WGS approach to check on actual off-target modifications (see point 6 below).
5. Apply the available suite of *in vitro* test methods to identify a 'superset of potential off-target cleavage sites' for a particular genome editing method (Akcakaya et al. 2018). This allows to check on the quality of the bioinformatics-based prediction of potential off-target sites. Consider if in vitro testing identifies potential activity at sites that are non-homologous to the genomic target sequence and thus not included in the prediction by bioinformatics tools.
6. Based on the above and a wider set of potential off-target sites, use targeted sequencing to detect actual off-target changes at the predicted genomic loci. Use targeted sequencing also to assess the genomic region which is genetically linked to the desired modification(s), i.e. in the (wider) vicinity of the target sequence for the intended changes.
7. Use WGS to scan for unintended changes in a non-biased way in case unlinked modifications have not been removed and the used method protocols are not optimised for high specificity or the method is known to be associated with off-target activity and no robust prediction of off-target activity is possible. The appropriate comparator is the genome sequence of the parental plant line which was subjected to modification by nGM approaches.
8. Assess whether any unintended changes might be of functional biological relevance. Consider if unintended changes might result in non-conservative nucleotide exchanges in coding sequences. Additionally consider whether unintended sequence changes might impact the regulatory function of the modified sequence.
9. Check whether it is possible to assess the significance of unintended changes in terms of biological effects. The following information might be helpful for such considerations: reference sequence data, further sequencing information from

different plant lines to assess the degree of natural variability of a particular genomic sequence and annotations of the functions of specific genomic sequences.

10. Targeted or untargeted phenotyping should be used to assess the possibility of adverse effects resulting from unintended modifications/off-target effects. In particular such an assessment should be required in case fast-track approaches are used to develop the final product (e.g. modification of elite lines, few or no crossbreeding possible or applied following the modification step). Such assessments should also be required in the case potential phenotypic effects are associated with identified unintended sequence changes which cannot be readily removed from the final product by crossbreeding (cf. results of points 4, 8 and 9).

5.4 nGM characteristics relevant for risk assessment considerations

5.4.1 Combination of different biotechnological and conventional methods

The scientific literature considered in this study demonstrates that in most cases specific nGMs are not used in isolation, but various biotechnological methods are combined in the different breeding processes to establish nGM applications. The following examples illustrate the various methodological relationships.

In many approaches GM technology is used at some point to establish intermediate or final products containing transgenic insertions. Typically such approaches are used to transfer and express the molecular tools necessary for the development of a variety of nGM applications. This includes e.g. expression of site-directed nuclease components for genome editing approaches, expression of transgenes in the modified rootstocks (or other parts) of plants established by transgrafting or during intermediate steps in the development of plants utilizing nGM approaches to speed up breeding cycles, e.g. accelerated breeding (ZHANG et al. 2010). For haploid induction, reverse breeding and accelerated breeding as well as for most products developed by SDN approaches for genome editing, the recombinant components are first integrated into the genome of the plant to be modified and then removed by segregation during later steps to derive the final breeding products.

Likewise nGMs may be used as technical tools to support the application of another nGM category. For instance genome editing can be used to knock out specific endogenous plant genes, e.g. to initiate early flowering as a tool for developing products by accelerated breeding (ZHANG et al. 2010), or to suppress meiotic recombination in plants which are used in reverse breeding applications (DIRKS et al. 2009). CRISPR-based systems in combination with DNA methyltransferases can be utilized for targeted modification of genomic methylation patterns to change the expression of targeted genetic elements (GUHA et al. 2017).

Genome editing of type SDN-3 is used to support the targeted insertion of transgenes at specific chromosomal loci and for molecular stacking of multiple transgenes (AINLEY et al. 2013, KUMAR et al. 2015). Such approaches may be similarly used for targeted insertion of cisgenic or intragenic constructs (AGES 2013).

SAUER et al. (2016b) and RIVERA-TORRES&KMIEC (2016) point out that ODM may be simultaneously applied with SDN techniques to make genome editing applications more efficient. Typically, the rate of sequence change by ODM is quite low, but is substantially increased, when DSBs are introduced in close vicinity to the ODM target site.

Other nGMs, such as agroinfiltration (VOGEL 2012) and/or the use of viral vectors for gene transfer and expression of method related components (BUTLER et al. 2016, LOZANO-DURAN 2016), are used as tools for transient gene expression in plant cells for two different purposes: (i) as a tool to study the effects of expression of a specific gene or genetic construct in a target crop, or (ii) as a tool to express molecules like dsRNAs or site-specific nucleases for further biotechnological modification of the respective crops, e.g. by RdDM or genome editing. Examples for (i) are the use of agroinfiltration to study the effects of transgenes involved in fatty acid metabolism (GRIMBERG et al. 2015), other examples are provided in VOGEL (2016). Examples for (ii) are approaches for the expression of site-specific nucleases as well as of donor DNA constructs required for SDN-2 and SDN-3 applications to initiate genome editing in the target plants (BALTES et al. 2014). In addition new approaches are developed to use viral vectors for plant modification in the environment, relying on insects to disseminate the viral vectors in the field (DARPA 2016, REEVES et al. 2018).

nGMs such as CENH3-mediated haploid induction (HI) were developed for the fast production of homozygous lines from a heterozygous parent without the need for lengthy back-crossing cycles. The method induces the *in vivo* production of haploid offspring from crosses between a haploid inducer line and a wildtype parent. Double haploid plants containing two identical sets of chromosomes can then be generated from the haploid lines in a second step. Haploid induction can be used to e.g. produce homozygous plant lines from genome edited plants (GURUSHIDZE et al. 2017). However, CENH3-mediated haploid induction could be applied as a general tool to speed up all breeding activities by substituting time-consuming back-crossing steps with the faster HI approach.

As already mentioned, conventional methods are typically used in all nGM approaches. Particular methods, e.g. *in vitro* culturing of isolated plant cells or tissues or protoplast technology, are associated with a different potential for inducing unintended modifications, especially the introduction of random genetic changes unrelated to the intended modifications (FILIPECKI&MALEPSZY 2006).

5.4.2 **Depth of intervention**

Genome editing applications of SDN-1 type introduce small sized, random sequence changes or even point mutations at targeted genomic locations. Due to the characteristics of the changes introduced by SDN-1 applications, they were compared with plants carrying spontaneous mutations or plants produced by classical mutagenesis (PAUWELS et al. 2014). However, spontaneous mutations and classical mutagenesis are neither directed nor targeted. Both widen the genetic diversity of plants in the first step and then breeders select plants with desired phenotypical modifications in a second step. As outlined below, certain SDN-1 applications, particularly applications to introduce multiple modifications at different genomic targets, can result in substantial metabolic reprogramming; this is generally overlooked when SDN-1 applications are merely judged by the small extent of genetic change introduced at single target sites.

Analysis of current developments show that several SDN-1 type applications aim to simultaneously introduce modifications (i) into multiple alleles, (ii) into all members of a gene family or (iii) into different functional genes, also called multiplexing (KHATODIA et al. 2016, PAUL III&QI 2016). In particular CRISPR-based systems for genome editing provide a

platform to achieve fast and efficient multiplexing in plants or other organisms (LOWDER et al. 2015, QI et al. 2016a, ZETSCHE et al. 2017, ZHANG et al. 2016e).

Proof of concept studies for multiplexed approaches with different site-directed nucleases were conducted in various crops, including maize (QI et al. 2016a), rice (XU et al. 2016b) and wheat (GIL-HUMANES et al. 2017a, WANG et al. 2014a). In rice up to 21 different target genes were modified in a single step (LIANG et al. 2016). In a recent study in wheat 35 different alpha-gliadin genes out of the 45 genes present in a wildtype line were knocked out using a multiplexed approach (SANCHEZ-LEON et al. 2018). SANCHEZ-LEON et al. (2018) suggest that multiplexed genome editing approaches can provide a route to develop low gluten wheat, something which has not been achieved by traditional plant breeding and mutagenesis approaches so far.

In the initial phase most genome editing applications addressed single genomic targets, i.e. single genes or all alleles of single genes. However, modifying complex polygenetic traits, like the gliadin content in wheat, requires simultaneous modification of multiple different genomic targets. For a significant number of multiplexed genome editing approaches no comparable products are available yet by conventional approaches. Conventional approaches were used only in few cases, such as a TILLING approach to introduce multigenic powdery mildew resistance (ACEVEDO-GARCIA et al. 2017). Therefore mostly no history of safe use is available for products of multiplexed applications of genome editing.

Further examples of multiplexed genome editing approaches address environmental stress response, plant development and composition:

- Knock-out of transcription factors CBF1/2/3, that directly regulate cold responsive genes in Arabidopsis (Shi et al. 2017b) (Zhao et al. 2016)
- Targeting of six of the fourteen PYL ABA receptor genes in Arabidopsis to assess their functional importance e.g. for root elongation and plant growth (ZHANG et al. 2016e)
- Knock-out of two ALCATRAZ (ALC) homoeologs involved in regulation of seed shattering of mature fruits in oilseed rape (Braatz et al. 2017)
- Knock-out of four closely related rice MPK genes essential for rice development (Minkenberg et al. 2017)
- Knock-out of three flowering suppressor genes that negatively control the heading date of rice varieties (LI et al. 2017f)
- Targeted mutagenesis of the three delta-12-desaturase (FAD2) genes to modify oil composition in camelina (Morineau et al. 2017) (Jiang et al. 2017)
- Targeted mutagenesis of the FAD2-1A and FAD2-1B genes to establish soybean varieties low in polyunsaturated fatty acids (Haun et al. 2014)

CHARI&CHURCH (2017) assume that the current approaches are only a first step to future large scale engineering of metabolic pathways and improved resistance to disease and environmental stress. They envision the application of extensive, but highly specific multiplexed genome editing in target organisms with the help of template DNAs, either fully

synthetic or extensively remodeled by MAGE ("multiplexed automated genome engineering") in a prior step (WANG&CHURCH 2011). Until now MAGE was not applied directly to plants.

The phenotypic outcomes of complex multiplexed interventions may not be fully predictable based on the available current information. In those cases further information and testing is necessary, e.g. based on the existing framework of GMO risk assessment. In addition presentations at a recent conference (OECD 2018) indicated that the overall efficacy of multiplexed editing approaches is still quite low. Low efficacy of approaches however could compromise their specificity and the low relative frequency of unintended changes. Since with multiplexing several different modified genes need to be retained in the final breeding product, the removal of unintended modifications through crossbreeding is more difficult to achieve. Thus, a sufficient molecular and phenotypic characterization is required to assess the effects of the genetic modifications on physiological functions. These considerations are not specific for multiplexed genome editing, but apply likewise to all nGM approaches resulting in complex and novel types of outcomes, e.g. modifications that result in manifold changes of gene expression in the respective plants or approaches for *de novo* domestication (see Chapter 5.2.4).

5.5 Risk assessment for nGM crops according to the EU regulatory framework

Until the ruling of the European Court of Justice (ECJ 2018) considerable legal uncertainty remained concerning the regulatory status of nGM applications, genome editing in particular (JONES 2015). Consequently it was also unclear whether risk assessment requirements for GMOs according to Directive 2001/18/EC would apply for nGM plants or not.

The ECJ ruled that organisms obtained by mutagenesis are GMOs and in principle subject to the obligations of Directive 2001/18/EC (ECJ 2018). The Court considered that the risks of the use of new techniques of mutagenesis might prove to be similar to those resulting from the release of GMOs developed by transgenesis. Indeed, many of the risk hypotheses e.g. considered by EFSA for GM plants (EFSA 2010, EFSA 2011) are also relevant for nGM plants with traits directed to increase environmental fitness to abiotic stress, diseases or pests, as well as traits for changed composition and herbicide resistance. The ECJ also referred to the novelty of nGMs, i.e. their lack of a long safety record, and their potential to produce GMOs at a significantly faster rate compared with methods of conventional mutagenesis. The Court's ruling is based on a legal analysis of the current regulatory framework in the EU, i.e. Directive 2001/18/EC. It concludes that applications of genome editing should undergo a premarket risk assessment and be subject to risk management as appropriate.

The Court ruling was met with content by some regulators, NGOs and some legal scholars as well as with sharp critique from agroindustry, biotechnology developers and some scientists. The latter called upon policy makers to amend Directive 2001/18/EC to exclude genome editing applications from regulation (PURNHAGEN et al. 2018, URNOV et al. 2018). Preliminary proposals towards this have already been submitted by the Netherlands, but have been met with mixed enthusiasm and support by other Member States. However, it remains to be seen whether any additional initiatives to amend Directive 2001/18/EC will be submitted in the near future. From a risk assessment point of view excluding genome editing

as well as other nGM applications from biosafety regulation right now would have significant consequences for the standard and quality of assessment which is provided for these applications: Other EU sectoral regulations which apply to all agricultural and food products, among others the EU Novel Food Regulation No (EU) 2015/2283 or the regulatory requirements for registration of plant varieties in EU or national catalogues according to Directive 2002/53/EC, fail to provide for a breadth and standard of risk assessment comparable with the requirements according to the respective biosafety frameworks (SPRANGER 2017) (ECKERSTORFER et al. 2019a).

5.6 Towards a case-specific framing of risk assessment for nGM applications

At present risk assessors and regulators face a number of challenges when considering which specific biosafety issues need to be addressed for nGM applications.

One major challenge is that the fields of nGMs in general and genome editing in particular are complex and rapidly developing. The overall range of such nGMs is very broad and expanding rapidly. The various methodologies used for crop modification aim at different breeding objectives and thus result in products with significantly different traits and characteristics. A common RA framework for all nGM plants therefore needs to take into account the range of methods used and the range of traits introduced. Not all plants developed by a particular nGM approach will be associated with a similar level of risk. Consequently potential risks of a nGM plant have to be considered in a case-specific manner, taking into account the characteristics of a particular nGM approach and the developed traits (SAM 2017).

Certain nGMs such as reverse breeding are applicable to selected plant species only and help to exploit the genetic diversity available rather than to generate genetic variability (SCHAART et al. 2016). Other nGMs like genome editing can be applied very broadly to all major annual crops and forest trees, and their respective genomes can be specifically targeted to introduce a variety of different traits. At present, the range of possible new traits and the crops that can be targeted seem to be constrained mostly by the limited knowledge of functional genomics and crop biology (SCHEBEN et al. 2017).

The level of risk associated with a certain nGM plant depends significantly, but not exclusively on the effects of the modified trait(s) on the overall characteristics of the modified plant species (DUENSING et al. 2018). With regards to the effects of the modified traits the RA needs to consider intended effects, as well as any unintended or unforeseen consequences of the expression of these modified trait(s). Three categories of nGM plants can be distinguished with respect to the target traits:

1. nGM plants with trait(s) which are related to traits occurring in similar crops which are produced by conventional approaches and which are used without adverse effects for comparable purposes. Typically these nGM plants will not contain non-native genes or genomic changes, that are not yet present in cultivated populations of the plant species (SCHAART et al. 2016). Several examples for this category are available, including herbicide resistant plants, plants with altered composition and plants resistant to e.g. fungal pathogens. The experience available with conventional plants harboring comparable traits can be used to judge whether plausible risks due to the specific traits may be expected.

2. This category comprises nGM plants with traits similar to those established in GM plants, e.g. herbicide resistance, disease resistance or insecticidal traits. For this category of nGM plants similar approaches for risk assessment to those implemented for the respective GMOs should be applied. Previous experiences with the assessment of such GMOs should be taken into account for the development of risk assessment approaches specifically adapted to the characteristics of nGM plants.
3. This category comprises traits which could not yet be established by conventional or other biotechnological methods. This category contains only novel, i.e. new and untried, traits developed by nGMs, e.g. through multiplexed approaches of genome editing resulting in complex physiological changes. A comprehensive RA should be conducted for these nGM plants, based on a case-specific problem formulation.

Our review of the available literature indicates that a wide range of nGM plants with novel traits is currently being developed for future agricultural use. Typically prior knowledge regarding safe use of these nGM plants is insufficient and the available information related to physiological functions of the modified genes and the effect of the specific modification(s) is limited.

Some of the novel traits will be based on multiple genetic modifications with possible complex impacts on metabolism and phenotype. Emerging methods, e.g. for multiplexed genome editing, simplify the rapid and simultaneous modification of multiple genome targets. Multiplexing increases the range of phenotypic changes that can be achieved at once, but also the depth (i.e. the extent) of molecular and physiological intervention. The present capacity of other biotechnological or conventional methods to achieve similar outcomes is limited. Typically no history of safe use is available for nGM applications and that increases uncertainty as to whether unintended effects may be associated with a particular application. Thus, the novelty status of traits developed with nGMs is a crucial factor regarding the risk assessment of nGM plants (HCB 2017).

However, possible risks are not restricted to nGM plants with novel traits. Experience with either conventional plants or GM plants indicate that plausible risk hypotheses may also apply to many of the nGM plants currently being developed to express traits that are not novel. Two examples illustrate the range of environmental risks: (i) In the case of resistance of nGM plants to abiotic stress, e.g. drought (SHI et al. 2017a, ZHANG et al. 2016b) or salinity (DUAN et al. 2016), possible environmental risks related to the outcrossing of such traits into related species need to be addressed; (ii) In case of HR nGM plants compositional changes through herbicide application as well as residues from cocktail mixes of pesticides need to be assessed for food and feed safety while indirect risks related to e.g. changes in weed management need to be addressed in terms of environmental safety.

The following aspects should be considered for the case-specific framing of a risk assessment of nGM plants, no matter whether the trait is novel or known: (i) the knowledge available for the targeted genomic locus and the impact (ii) of the (genetic) modification and (iii) of the expression of the modified trait on the physiology and phenology of the nGM plant. Our findings indicate that very diverse cellular mechanisms and functional pathways are involved in different groups of nGM applications: HR plants, plants with resistance to diseases, plants with changed composition and plants with increased resistance to environmental stressors and altered morphology or reproduction. Significant differences

concerning relevant risk issues also exist between individual applications in those groups. The level of new information required to assess the respective issues should consider the extent of scientific knowledge and experience available for the specific nGM plants and traits.

It is doubtful that the overall experience with traits derived from classical mutagenesis can provide a safe history of use for all novel traits developed e.g. by SDN-1 applications. It is reassuring that in the past no plant safety issues emerged for the mutants developed by classical mutagenesis (DUENSING et al. 2018). However, this conclusion cannot simply be extrapolated to all SDN-1 traits, because, on the one hand, a fair number of these traits are novel, and on the other hand, adverse effects may not always be removed during further crossbreeding steps and selection – steps which are indispensable in applications of classical mutagenesis. Without having analysed possible effects caused by a particular genetic change a general assumption of safety for all SDN-1 applications lacks a robust scientific basis.

Novel traits may be developed in a very specific manner, e.g. by genome editing approaches. However, it should be noted that the level of specificity of an nGM approach *per se* does not provide an adequate measure of the level of risk associated with the respective trait.

However, the level of specificity should be considered during the assessment of unintended effects related to the methods employed. Again, the specific characteristics of the respective nGM methods (i.e. how they work and at which stage they are used) as well as their level of specificity have to be considered in a case-specific manner. The need for such an approach is illustrated by the spectrum of available methods for genome editing, including ODM and the many different applications of the CRISPR system. As mentioned, these methods introduce different modifications including (i) small random mutations at specific genomic loci (SDN-1), (ii) directed, but typically small sequence changes at specific genomic locations (SDN-2 and base-editing) and (iii) targeted insertion of exogenous genetic constructs and transgenes (SDN-3). In addition, specific epigenetic changes can be achieved by modifying the methylation pattern. Different levels of off-target activity and different outcomes are associated with the different approaches. Even if the number of off-target mutations may be lower for genome editing approaches compared to some approaches for random mutagenesis, especially when disregarding subsequent screening and breeding steps, they should not be neglected. A case-specific analysis of off-target activity can provide useful indications whether potential adverse outcomes may be expected (ZHAO&WOLT 2017). This approach should not just rely on predictions by bioinformatics, since these tools might not be robust enough yet (CAMERON et al. 2017, ZISCHEWSKI et al. 2017). Additional analytical testing is required and a range of approaches is available for focused as well as unbiased genome-wide assessment (AGAPITO-TENFEN et al. 2018).

Schemes to develop nGM plants typically involve a combination of different technologies. Most nGM approaches also involve GM technology at certain (intermediate) steps and/or techniques of cell and tissue cultivation and regeneration, e.g. protoplast technology, which cause an elevated level of random genetic change (WOLT 2017). Therefore, genome editing approaches should not be solely judged by the specificity of their mechanism, e.g. the characteristics of the used type of site-directed nuclease. On the contrary, a comprehensive view is required to consider the potential of the overall development process to either induce unintended genetic changes or to remove unwanted mutations during downstream steps.

Some nGMs like genome editing can speed up breeding processes significantly, e.g. by direct modification of elite lines, which in turn can impair the likelihood to detect and remove those unintended genetic changes, which are not genetically linked to the intended modification, when the final product is established.

In our opinion a general assessment framework should be implemented for nGM plants, which is addressing the characteristics of each particular nGM plant, its traits and the consequences of unintended effects. It would incorporate the following elements, some of which were recommended to be used in a case-specific way by other authors (HUANG et al. 2016, RICROCH et al. 2016):

- Case-specific risk assessment requirements, which take into consideration the nature of the developed traits, unintended consequences of the introduced modifications, the available experience with comparable products and relevant protection goals specified by the respective countries.
- Appropriate molecular characterization, to assess amongst other things whether any transgenic inserts are unintentionally present in final nGM products and to determine the presence of off-target modifications and other unintended genetic changes, which might result in adverse phenotypic effects.
- Phenotypic characterization to specifically test parameters related to plausible risk issues associated with particular nGM plants, that are not covered by other existing legislation applicable to nGM plants, e.g. plant variety registration, food safety and others (Spranger 2017).

For a robust, yet case-specific characterization of unintended effects associated with nGM plants we recommend that risk assessors apply the 10 step approach outlined in chapter 5.3.6.

The existing regulatory framework in the EU for GMOs includes requirements for a scientific risk assessment conducted by EFSA (AGAPITO-TENFEN et al. 2018). The currently applied assessment approach is based on a case-specific problem formulation according to the principles and the general process laid out in Directive 2001/18/EC (EFSA 2010).

EFSA has already conducted an initial evaluation for some nGM applications, i.e. plants developed through cisgenesis, intragenesis and SDN-3 type applications of genome editing, as to whether and how specific risk issues should be considered for such nGM plants (EFSA-PANEL ON GMOS 2012a, EFSA-PANEL ON GMOS 2012b). These studies should be revisited and used as input to develop robust risk assessment approaches for such applications. Similar evaluations need to be conducted for all nGM applications included in the ruling of the ECJ, particularly for emerging technologies like CRISPR-based genome editing which can be applied in many ways and with many variants. The experience available with risk assessments for nGM products according to the existing worldwide regulatory frameworks for biosafety should be taken into account during this exercise. However, at present the experience with such assessments is quite limited (WOLT 2017), partly due to the decisions of a number of countries not to regulate some nGM plants (ECKERSTORFER et al. 2019a, WALTZ 2018). Against this background of limited knowledge and experience we recommend that a case-specific risk assessment is conducted for nGM plants to address all relevant risk issues accordingly.

6 Conclusions

A broad range of nGMs including genome editing is currently available and further methods allowing complex modification of plants are rapidly being developed. They are used to develop nGM plants with different traits and characteristics, which will be associated with different levels of risk. With respect to intended traits three categories of nGM plants can be distinguished (apart from further considerations regarding e.g. crop type, purpose of application and use, etc., that have to be taken into account additionally):

1. nGM plants with traits and usage known from conventional approaches and without adverse effects
2. nGM plants with traits known from established GM plants, e.g. herbicide resistance or disease resistance, and associated with comparable risk issues
3. nGM plants with traits which have not yet been established and thus need to be considered as novel

Our study shows that nGM applications may be found for all three categories; the same applies for all sub-classes of genome editing (SDN-1, SDN-2 and SDN-3). Therefore, regulation and risk assessment has to acknowledge that all nGM groups will be comprised of a mix of applications with lower as well as higher uncertainty regarding their level of risk/safety. In addition nGM applications are fairly new and only a few plants developed with these methods have been risk assessed for cultivation purposes so far. Against this background of insufficient knowledge and experience for a variety of applications, we argue that a general framework for biosafety oversight is further implemented for nGM plants, based on a case-specific risk assessment incorporating the following elements:

- Case-specific risk assessment requirements taking into account (i) the nature of the developed trait, (ii) unintended consequences of the modification introduced, (iii) the available experience with comparable products and (iv) relevant protection goals specified by the respective countries.
- Appropriate molecular characterization to assess among other things (i) the unintentional presence of any transgenic inserts in the final product and (ii) the presence of off-target modifications and other unintended genetic changes, which might result in adverse phenotypic effects.
- Phenotypic characterization to specifically test parameters related to plausible risk issues associated with a particular nGM plant.

This will require that the existing guidance for risk assessment of GMOs as established in the EU by EFSA be reviewed as to whether it is suitable, sufficient and appropriate for specific types of nGM applications. Specific guidance needs be developed which enables risk assessors to focus their attention and resources on issues of concern specific for the different applications and to use established and emerging tools for their assessment.

With a view to the development of ever faster and ever more complex and sophisticated breeding approaches this will not be an easy task. However, in our opinion the efforts will be worthwhile from a safety perspective and a better alternative to exempting nGM applications from biosafety assessments altogether.

7 Annexes summarising the results of a review of scientific literature on recent nGM applications

7.1 Annex 1: CRISPR/Cas Applications

Table 7: CRISPR-based applications retrieved from recent scientific literature (2016-2017)

(Cat. - Categories Column 1: **(1)** Cas9 for SDN1, including multiplexing, Cas9 RNPs, transient expression, gRNA RNP, PTG = polycistronic tRNA-gRNA (PTG) (29), VQR variant (111), spCas9 (42); **(2)** Cas9 for SDN1 with Cas9 from different species; **(3)** Cpf1 for SDN1; **(4)** Cas9 & Cpf1 for SDN1; **(5)** SDN2; **(6)** SDN3 (and SDN1 or -2); **(7)** nCas9; **(8)** nCas9 coupled to cytidine deaminase)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
1	Cas9 (codon-optimized *Streptococcus pyogenes* Cas9)	Alfalfa (*Medicago sativa*) legume	Albino phenotype	To disrupt an endogenous gene, here phytoene desaturase (*Mt*PDS)	**Methodology:** To test CRISPR/Cas system in (forage) legumes	(MENG et al. 2017)
1	Cas9 (Cas9 fused to GFBSD2)	Apple	Clear and partial albino phenotypes	Knock-out of phytoene desaturase (PDS) gene using 3 sgRNAs and one 18bp tru-sgRNA Using Cas-GFBSD2 fusion gene (with green fluorescence protein and brastcidin resistance gene) allows to monitor and select Cas9-expressing cells via GFP	**Methodology:** First study in apple using CRISPR/Cas9	(NISHITANI et al. 2016)
1	Cas9 (and dual-sgRNA)	*Arabidopsis thaliana*	Modulation of onset of flowering	Creation of targeted inversions (proof of concept/ approach) Cross-validation system: FLOWERING TIME (*At*FT) and TERMINAL FLOWER 1 (*At*TFL1) loci, florigen genes with opposing functions (FT promotes flowering, whereas TFL1 represses flowering)	**Methodology work**: To introduce targeted DNA inversions of interest for functional studies and crop improvement	(ZHANG et al. 2017a)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
1	Cas9 (egg-cell specific)	*Arabidopsis thaliana*	Disruption of female gametophyte development Variegated and serrated leaves	Knock-out & knock-down of RRP42 (a subunit of the exosome complex) CRISPR/Cas with egg-cell specific promoter	**Basic research:** To investigate and demonstrate the role of RRP42 for the development of female gametophytes and mesophyll cell morphogenesis	(YAN et al. 2017)
1	Cas9	*Arabidopsis thaliana*	Not relevant	DSB (double strand break) in cruciferin 3 (CRU3) and protoporphyrinogen oxidase (PPO) genes	**Basic research:** To better understand NHEJ pathways and factors (other than the known KU and PARPs)	(SHEN et al. 2017a)
1	Cas9	*Arabidopsis thaliana*	Bialaphos herbicide resistance and hairless leaves	To design a vector and reporter system for CRISPR/Cas activity. Target: disabled bialaphos resistance (bar) transgene and endogenous glabrous1 (GL1) - needed for formation of trichomes	**Methodology**: For testing of CRISPR/Cas9 activity in Arabidopsis	(HAHN et al. 2017)
1	Cas9 (germline-specific)	*Arabidopsis thaliana*	Not relevant	Targeted genes: APETALA1 (AP1) (AT1G69120) and TRANSPARENT TESTA 4 (TT4) (AT5G13930), each targeted at two different positions within their coding regions Germline-specific Cas9 system SPL genomic expression cassette)	**Methodology:** To achieve high level of heritability, using germline-specific Cas9 system (GSC) for Arabidopsis gene modification in male gametocytes	(MAO et al. 2016)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
1	Cas9	*Arabidopsis thaliana*	Cold stress response and cold acclimation	Knock-out of CBF genes (C-repeat binding factors), double and triple mutants	**Basic research:** To understand exact functions of CBF1,2 and 3	(JIA et al. 2016b)
1	Cas9	*Arabidopsis thaliana*	Not relevant	Knock-out of AUXINBINDING PROTEIN1 (ABP1) gene by first mutation, and second by large deletion	**Methodology:** Visual screen for Cas9-free T2 seeds with red fluorescence (adding a cassette that enables the expression of the mCherry gene under the control of a strong promoter. The mCherry cassette allows to visually select Cas9-free plants in the T2 generation)	(GAO et al. 2016)
1	Cas9 (meiosis-specific)	*Arabidopsis thaliana*	Phenotypes of a) clustered leaf trichomes and b) increased trichomes ant leaf edge (as visual markers)	Target genes: ENHANCER OF TRY AND CPC 2 (ETC2), CAPRICE (CPC), and TRIPTYCHON (TRY) genes (Fig. 1b). The try cpc double mutants have an easily screenable phenotype of clustered leaf trichomes (Wang et al. 2015) and the etc2 try cpc triple mutants also have an easily screenable phenotype of increased trichome development at the edges of leaves and on petioles	**Methodology:** Improve CRISPR/Cas editing for Arabidopsis, as the generation of homozygous or bi-allelic mutants in the first (T1) generation is inefficient. Specific promoters used to drive the expression of Cas9 during meiosis to maximize the efficiency of recovering heritable mutants in T1 plants	(EID et al. 2016)
1	Cas9	*Arabidopsis thaliana*	a) albino phenotype b) 'double flower'	Creation of high efficiency CRISPR vector for plants, pKAMA-ITACHI Red (pKIR),	**Methodology:** Development and optimization of vector, as a tool to induce heritable mutations highly	(TSUTSUI&HIGASHIYAMA 2017)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
			c) single non-fertile sperm-like cell (traits as reporter)	harboring the RIBOSOMAL PROTEIN S5 A (RPS5A) constitutive promoter (throughout development, beginning in egg cells) to drive Cas9 Target genes for knock-out: to test promoters, efficiencies, transmission: PHYTOENE DESATURASE 3 (PDS3), AGAMOUS (AG), DUO POLLEN 1 (DUO1)	efficiently in *A. thaliana* and other species	
1	Cas9	*Arabidopsis thaliana*	Altered cold-response	Knock-out loss-of-function mutants of transcription factors CBF1/2/3, that directly regulate cold responsive (*COR*) genes CBF1/DREB1B, CBF2/ DREB1C CBF3/DREB1A	**Basic research:** To investigate the molecular mechanisms of the cold responsive network in *A. thaliana* to help elucidate the mechanisms underlying adaptation at the molecular level (trade-off between cold tolerance and plant growth)	(SHI et al. 2017b)
1	Cas9	*Arabidopsis thaliana*	Stress tolerance (low temperature, drought, salt tolerance)	Targeting UGT79B2 and UGT79B3 genes, which can be strongly induced by various abiotic stresses Knock-out: increased stress sensitivity. (also using RNAi)	**Basic research:** Into stress response and stress tolerance. To further understand the role of specific UDP-glycosyltransferases (UGTs) in modulating anthocyanin biosynthesis and abiotic stress tolerance	(LI et al. 2017c)
1	Cas9	*Arabidopsis thaliana*	Cold & freeze tolerance Salt tolerance	Knock-out of: CBF1, CBF2 and CBF3 – individually, as doubles and as triples	**Basic research:** Functional genomics – to investigate and demonstrate the critical role of CBF transcription factors in cold	(ZHAO&ZHU 2016)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
			Seedling development		acclimation and freezing tolerance	
1	Cas9	*Arabidopsis thaliana*	ABA insensitive phenotype	Targeting six of the fourteen PYL families of ABA receptor genes (Abscisic acid (ABA), is a sesquiterpenoid phytohormone)	**Methodology:** Multiplexing (CRISPR/Cas9 system that allows the co-expression of six sgRNA modules in one binary vector (under 3 different promoters)	(ZHANG et al. 2016e)
1	Cas9 (deletions) (UBQ10 promoter)	*Arabidopsis thaliana*	Alteration of flowering times.	Proof of concept: Large fragment deletions for knock-out or knock-down: 1) Regulatory intron in AGAMOUS gene (flower development gene) 2) ELF6 gene (early flowering), REF6 (relative of early flowering gene) and SEP3	**Methodology:** Multiplexing for knock-out of multiple genes and generation of heritable large fragment deletions. Improving efficiency of CRISPR/Cas system	(YAN et al. 2016)
1	Cas9	*Arabidopsis thaliana*	Amongst other: reduced capacity to actively take up external sucrose in roots.	Knock-out and loss-of-function of: cell wall invertase 1 gene (*At*CWIN1) – frameshift	**Basic research:** To explore the role of cell wall invertases (CWINs) in sucrose transport in roots and leaves and during *Botrytis cinera* infection (a necrotrophic fungus)	(VEILLET et al. 2016)
1	Cas9	*Arabidopsis thaliana*	Potyvirus resistance	Loss-of-function mutations of the eIF(iso)4E locus (eukaryotic translation initiation factor gene), known to give rise to potyvirus resistance if homozygous	**R&D:** To provide a show case for application in crops to achieve transgene free potyvirus resistance with heritable, homozygous mutations in self-pollinating species in the T2 generation	(PYOTT et al. 2016)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
1	Cas9 multiplexing	*Arabidopsis thaliana*		Assessment of efficiency and specificity of 7 out of the 11 members of GOLVEN (GLV/RGF/CLEL) gene family (encode peptides that regulate several aspects of root stem cell development)	**Methodology:** Multiplexing: Assembled gRNA expression units into "stackable arrays" targeting up to 14 sites at once	(PETERSON et al. 2016)
1	Cas9 with truncated gRNA (tru-gRNA/Cas9 combination)	*Arabidopsis thaliana*	Altered stomatal closing in response to environmental conditions	Utilising GFP-Cas9 co-expression to select Cas9 active cells Generated new alleles for OST2, (a proton pump in Arabidopsis) for stress resistance	**Methodology**: Optimising the CRISPR/Cas system and increasing efficiency by using truncated gRNA (tru-gRNA)/Cas9 combination with meristematic- and reproductive-tissue-specific promoters	(OSAKABE et al. 2016)
1	Cas9	*Arabidopsis thaliana* and a wild tobacco (*Nicotiana attenuata)*	Not relevant	Knock-out: *At*SH3P3 gene (AT4G18060) in *A. thalina*, NaAOC gene in *N. attenuata*.	**Methodology:** To provide new binary vector system designed for fast throughput easy cloning of sgRNAs, Cas9 coding and exchangeable promoter sequences to enable targeted large-scale knock-out lines	(KIM et al. 2017b)
1	Cas9 fragment deletions	Barley *(Hordeum vulgare* cv. "Golden Promise")	Not relevant	Knock-out and loss-of-function: creating indels and fragment deletions in the putative endo-N-acetyl-b-D glucosaminidase (ENGase) gene	**Methodology:** To show barley is a good candidate for CRISPR/Cas with different forms of transformation. To study the modification of N-glycans in cereal grains	(KAPUSI et al. 2017)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
1	Cas9	*Camelina sativa*	Altered C18 fatty acids desaturation profile (less saturated, more oleic acid)	Selective, targeted mutagenesis of the three delta-12-desaturase (FAD2) genes (*C. sativa* has three closely related expressed sub-genomes) Using two sgRNAs to target the three FAD2 gene sequences.	**Methodology/Basic research:** To investigate the possibility of creating a large collection of combinatorial mutants by engineering gene dosage, particularly in polyploid genomes	(MORINEAU et al. 2017)
1	Cas9	*Camelina sativa* & *Arabidopsis thaliana*	Enhancement of fatty acid composition in seed (increase of oleic acid content to 50%)	Targeted all three homoeologous FAD2 genes in the allohexaploid *Camelina*	**R&D:** To improve seed oil composition	(JIANG et al. 2017)
1	Cas9	*Chrysanthemum morifolium* (1st time)	Knock-out of reporter gene	Knock-out of yellowish-green fluorescent protein (transgene)	**Methodology:** To opening up research and to test methodology in a hexaploid large genome plant with limited genome information	(KISHI-KABOSHI et al. 2017)
1	Cas9	Cotton (*Gossypium hirsutum* L.)	Not relevant	Targeting: Cloroplastos alterados 1 (GhCLA1) and vacuolar H^+-pyrophosphatase (GhVP) genes (no bi-allelic mutants found)	**Methodology:** First time application in cotton (proof of principle). To show CRISPR/Cas may be used in cotton, for advancing cotton functional genomic research and to increase the potential for cotton molecular breeding	(CHEN et al. 2017)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
1	Cas9	Cucumber (*Cucumis sativus* L.)	Broad virus resistance: ipomovirus potyvirus	Knock-out: To disrupt the function of the recessive eIF4E (eukaryotic translation initiation factor 4E) gene.	**R&D:** To develop transgene-free immunity to cucumber vein yellowing virus infection and resistance to the potyviruses: zucchini yellow mosaic virus, papaya ring spot mosaic virus	(CHANDRASEKARAN et al. 2016)
1	Cas9 RNPs	Grape (cultivar Chardonnay) & Apple (Golden Delicious)	Disease resistance (powdery mildew & fire blight)	Knock-out of *MLO*-7, a susceptible gene in order to increase resistance to powdery mildew in grape; DIPM-1, DIPM2, and DIPM-4 in the apple to increase resistance to fire blight	**Methodology:** To demonstrate that direct delivery of CRISPR Cas9 RNPs (ribonucleoproteins) to protoplasts facilitates targeted gene editing and allows for the generation of DNA-free genome edited grapevine and apple plants	(MALNOY et al. 2016)
1	Cas9	Grape (*Vitis vinifera* L. cv. Chardonnay)	Not relevant	L-idonate dehydrogenase gene (IdnDH)	**Methodology:** Show CRISPR/Cas editing works in grape	(REN et al. 2016)
1	Cas9	Grape (*Vitis vinifera* L., cv. Neo Muscat)	Albino phenotype	Knock-out: Phytoene desaturase (*Vv*PDS) gene	**Methodology:** To show CRISPR/Cas9 works in grape. To compare efficiency with different transformation methods.	(NAKAJIMA et al. 2017)
1	Cas9	Grapefruit/citrus	Resistance to bacterial citrus canker	Modify the canker susceptibility gene *Cs*LOB1.	**Basic research/R&D:** To easily obtain disease resistance	(JIA et al. 2017)

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1	Cas9	Grapefruit/citrus	Reduced disease-susceptibility (towards canker-resistance)	Modify the PthA4 effector binding elements (EBEs) in the *Cs*LOB1 Promoter of *Cs*LOB1 (*Citrus sinensis* Lateral Organ Boundaries)	**Basic research/R&D:** Towards canker resistance via modification of *Cs*LOB1 is a susceptibility gene for citrus canker induced by the pathogenicity factor PthA4	(JIA et al. 2016a)
1	Cas9	*Lotus japonicus* (model legume)	Inactivation of Symbiotic Nitrogen Fixation	Inactivation of symbiotic nitrogen fixation (SNF) related genes, such as targeting three homologous leghemoglobin loci (LjLb1, LjLb2, LjLb3) and SYMRK (symbiosis receptor-like kinase).	**Methodology/Basic research**: To show CRISPR/Cas works in *Lotus japonicus* and can facilitate functional analysis of symbiotic nitrogen fixation (SNF) related genes.	(WANG et al. 2016b)
1	Cas9	Maize	Not relevant	Argonaute 18 (*Zm*Ago18a and *Zm*Ago18b) and dihydroflavonol 4-reductase or anthocyanin-less genes (a1 and a4) - each with members on two different chromosomes	**Methodology:** To test and evaluate a public sector system, the ISU maize CRISPR platform, for efficient and effective gene targeting	(CHAR et al. 2017)
1	Cas9	Maize	Albino phenotype (reporter trait)	Knock-out of maize phytoene synthase gene (PSY1)	**Methodology:** To show CRISPR/Cas can be successfully applied for genome editing in maize	(ZHU et al. 2016)

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1	Cas9 multiplexing (based on the tRNA-processing system from rice)	Maize	Not relevant	Simplex editing targets: three transcription factors: maize MADS gene (GRMZM2G059102), MYBR gene (GRMZM2G091201), and AP2 gene (GRMZM2G050851) Multiplex editing: maize RPL gene (GRMZM2G024838), PPR gene (GRMZM2G087226), and two reverse overlapping maize long non-coding RNAs	**Methodology:** To test and introduce a specific vector construction, sequence design and editing results of using the multiplex gene editing strategy based on the tRNA-processing system in maize	(QI et al. 2016a)
1	Cas9	Maize	Albino phenotype/photobleaching;	Knock-out of: *Zm*zb7 gene. Twelve sites with different expression levels in maize centromere/ pericentromere regions were selected	**Methodology/R&D:** To show that CRISPR/Cas9 is a robust and efficient tool for genome modification in both euchromatic and heterochromatic regions in maize	(FENG et al. 2016)
1	Cas9	*Medicago truncatula* (barrelclover - model legume)	Quantitative variation in nodulation	-	**Methodology:** CRISPR/Cas9 combined with Genome-Wide Association (GWA) to identify the genetic basis of ecological and economical important traits.	(CURTIN et al. 2017)
1	Cas9 multiplexing	Moss: *Physcomitrella patens* (model plant)	Not relevant	*Pp*KAI2L (KARRIKIN INSENSITIVE 2 LIKE) and AP2/ERF (APETALA 2/ERE binding factor) gene families	**Methodology:** To develop a transient system for simple and efficient targeting of multiple genes	(LOPEZ-OBANDO et al. 2016)

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1	Cas9	Oilseed Rape (*Brassica napus*)	Increased shatter resistance of seed	Knock-out: Targeting two ALCATRAZ (ALC) homoeologs (which contribute to seed shattering from mature fruits)	**Methodology/R&D:** To demonstrate the potential of CRISPR/Cas9 editing for simultaneous modification of different homoeologous gene copies in a polyploid species (here tetraploid)	(BRAATZ et al. 2017)
1	Cas9	Opium Poppy (*Papaver somniferum* L.)	Reduced BIAs production (eg morphines)	Knock-out of 4′OMT2, a gene which regulates the biosynthesis of benzylisoquinoline alkaloids (BIAs) - (e.g. morphine, thebaine)	**Methodology:** First time editing in poppy (medicinal aromatic plants). To show the ability of CRISPR/Cas to manipulate the biosynthesis of bioactive compound alkaloids for Next-Generation Metabolic Engineering	(ALAGOZ et al. 2016)
1	Cas9	Orchid (*Dendrobium officinale*) (also top-ranked Chinese medicinal herb)	GUS and green fluorescent protein as reporter genes	To select appropriate promoters (tested CaMV 35S & 3 other viral ones). Five target genes from the lignocellulose biosynthesis pathway (C3H, C4H, 4CL, CCR, and IRX) tested for editing by PCR method	**Methodology:** To develop CRISPR/Cas9 system for orchids, esp. finding right promoters	(KUI et al. 2017)
1	Cas9	Petunia (*Petunia* hybrid)	Albino phenotype	Targeting PDS gene (indels and fragment deletions)	**Methodology:** Establishing CRISPR/Cas9 for Petunia	(ZHANG et al. 2016a)
1	Cas9 gRNA RNP	Petunia (*Petunia* hybrid)	Not relevant	Targeting nitrate reductase (NR) gene	**Methodology:** To show RGEN (RNA-guided endonuclease) RNPs work in plant systems, esp. with protoplast systems	(SUBBURAJ et al. 2016) *

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1	Cas9	*Populus tomentosa*	Altered composition of secondary cell wall formation	Gene knock-out of *Pto*MYB156, encoding a R2R3-MYB transcription factor	**Basic research**: Functional characterisation of *Pto*MYB156, encoding a R2R3-MYB transcription factor involved in secondary cell wall formation/thickness/biosynthesis	(YANG et al. 2017)
1	Cas9	*Populus tomentosa*	Composition	myb115 mutant to decrease expression of PA biosynthetic genes (in parallel to upregulation by other method).	**Basic research:** Transcriptional regulation of proanthocyanidins (PAs) by MYB115	(WANG et al. 2017)
1	Cas9	Potato (*Solanum tuberosum*) (& *Arabidopsis*)	No specifics included	Knock-out of *St*MYB44 gene (*Solanum tuberosum* transcription factor gene MYB44) which regulates phosphate transport by suppressing *St*PHO1 expression	**Basic Research:** To understand the molecular basis of the complex responses of potato (*Solanum tuberosum* L.) to phosphate (Pi) deficiency stress	(ZHOU et al. 2017)
1	Cas 9 (transient)	Potato (*Solanum tuberosum*) – tetraploid	Altered starch quality	Knock-out of granule-bound starch synthase (GBSS) gene (targeted multiallelic mutagenesis in tetraploid plant, using protoplasts)	**Methodology and R&D:** To demonstrate transient presence of CRISPR/Cas will give rise to targeted genome editing in protoplasts of tetraploid potato (*Solanum tuberosum*) and can yield mutations in all four alleles in a single transfection	(ANDERSSON et al. 2017)
1	Cas9	Rice	Temperature induced male sterility for hybrid breeding	Knock-out of: Thermo-sensitive Genic Male Sterility gene (TGMS or TMS) for hybrid breeding (encodes the	**R&D:** For commercial application. To develop a system for producing commercial “transgene clean”	(ZHOU et al. 2016)

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				endonuclease RNase Z).	thermo-sensitive male sterile (TGMS) rice lines for hybrid breeding (TMS5 Editing System)	
1	Cas9	Rice	High amylose trait for health benefit	Mutating starch branching enzyme (SBE), genes for SBEI and SBEIIb	**R&D:** To show ability to alter amylose content	(SUN et al. 2017b)
1	Cas9	Rice	Knock-out of beta-glucuronidase (GUS)	1.6 kb beta-glucuronidase (GUS) gene removed, i.e. excision of transgenes	**R&D:** Creating marker-free plants, esp. for antibiotic-resistance marker genes	(SRIVASTAVA et al. 2017)
1	Cas9	Rice	8 agronomic traits: dense and erect panicle, grain number, major quantitative trait loci, grain length and weight, QTL associated with rice grain width and weight, heading date; erect panicle, loose plant architecture	Randomly selected agronomic genes: BADH2 (betaine aldehyde dehydrogenase 2), DEP1, Gn1a, QTL, GS3 (for), GW2 (), Hd1, EP3, and LPA1 Five genes are related to rice yield (DEP1, EP3, Gn1a, GS3, and GW2), one regulates plant architecture (LPA1), BADH2 has a relationship with rice fragrance and Hd1 with photoperiod	**R&D:** To demonstrate the potential of the CRISPR/Cas9 system for rapid generation of genetic diversity for crop breeding. To further explore QTLs (quantitative trait locus) and multiplexing	(SHEN et al. 2017b)

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1	PTG/Cas9 multiplexing	Rice	Extremely dwarfed and sterile	Knock-outs of MPK1 and MPK6 (Arabidopsis *At*MPK6 and *At*MPK4 orthologs, respectively) found to be essential genes for rice development by finding the preservation of MPK functional alleles Performed highly efficient targeting of four closely related rice MPK genes with PTG/Cas9	**Basic research/Methodology:** By using the polycistronic tRNA-gRNA (PTG) gene system and by efficiently co-expressing multiple RNAs for multiplex gene editing, the characterization of genes can lead to the discovery of new genes Multiplexing	(MINKENBERG et al. 2017)
1	Cas9	Rice	Significant decrease of the sucrose concentration in the mutant embryosacs, defective grain filling	Knock-out of sugar transporter *Os*SWEET11 gene	**Basic research**: To increase the understanding of post-phloem sugar transport during the early stage of rice caryopsis development in rice	(MA et al. 2017)
1	Cas9	Rice	Increased sensitivity to stress (drought, reactive oxygen species (ROS). Decreased ABA sensitivity	Knock-out, loss-of-function of: SAPK2 (SAPK2 = stress/ABA-activated protein kinase) upregulated by drought, high-salinity, and polyethylene glycol (PEG) treatments	**Basic research:** To elucidate the functional properties of SAPK2, which is part of a family of plant-specific protein kinases, the key regulators of hyper-osmotic stress signalling and abscisic acid (ABA)-dependent development in various plants	(LOU et al. 2017)

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1	Cas9	Rice	Early-maturing variety (with low photoperiod sensitivity)	Knock-out of: Hd2, Hd4 and Hd5, (three flowering suppressors in Ehd1-dependent photoperiodic flowering pathway) are major genes that negatively control the heading date of rice varieties	**Basic research:** To identify and alter gene regulation in order to create a rice cultivar with early-maturation and extremely low photoperiod sensitivity.	(LI et al. 2017f)
1	Cas9	Rice	Albino phenotype	Phytoene desaturase (*Os*PDS) - 22 T0 plants from previous work (Wang et al. 2015)	**Methodology:** To devise and test a cost-effective and sensitive methodology for CRISPR/Cas9-induced mutant screening technique based on conventional PCR, annealing at critical temperature PCR (ACT-PCR), for identifying mutants	(HUA et al. 2017)
1	Cas9	Rice	Not relevant (Grain yield in rice)	*Os*ROC5 and *Os*DEP1	**Methodology**: To test a mutation detection method based on single-strand conformational polymorphism (SSCP) to screen for homozygous or heterozygous mutants	(ZHENG et al. 2016)
1	Cas9 multiplexing	Rice	Improved grain weight	To test pyramid effect of knock-out of GW2, GW5 and TGW6, each mutant increasing grain weight	**Basic research**: To understand functions of genes involved in yield	(XU et al. 2016b)

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1	Cas9	Rice	Cold stress sensitive	Knock-down and -out of TCD10, creating a PPR gene mutant tcd10 (thermo-sensitive chlorophyll-deficient mutant 10) that exhibits the albino phenotype	**Basic research**: To understand genes involved in chloroplast development under cold stress	(WU et al. 2016b)
1	Cas9	Rice	Pollen tube burst after germination	Frameshift/loss-of-function mutations in RUPO. Identification of a novel CrRLK1L receptor kinase RUPO from rice	**Basic research:** Functional genomics	(LIU et al. 2016)
1	Cas9	Rice	Enhanced grain number, dense erect panicles, and larger grain size.	Four yield-related genes Gn1a, DEP1, GS3, and IPA1 (regulators of grain number, panicle architecture, grain size and plant architecture)	**Methodology**: To show that multiple regulators of important traits can be modified in a single cultivar by CRISPR/Cas9 and to reassess gene function	(LI et al. 2016c)
1	Cas9	Rice	Stress :response	Knock-out JAZ9 activity to understand its function in stress response	**Basic research:** Testing and improving applicability of CRISPR/Cas to induce homozygous mutations	(JANG et al. 2016)
1	Cas9	Rice	Albino phenotype	Knock-out of phytoene desaturase to study segregation of mutations in presence of Cas9	**Methodology**	(ISHIZAKI 2016)

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1	Cas9 VQR variant	Rice	Not relevant	The LPA1, LG1, and GL1-1 genes were selected for further genome editing using the VQR variant. Mutations of these genes result in increased tiller angle, loss of several specialized organs (laminar joint, auricle, ligule) and the disrupted formation of leaf cuticular wax	**Methodology:** To test an engineered Cas9 variant (with altered PAM) in rice	(HU et al. 2016)
1	Cas 9	Rice	Altered starch/sugar content in leaves	Knock-out of *Os*SPS1 and *Os*SPS11 (sucrose phosphate synthase)	**Basic research:** To understand the role of SPS in rice in the starch: sugar content ratio, with rice being a 'sugar leaf' plant, whilst Arabidopsis is 'starch leaf' plant.	(HASHIDA et al. 2016)
1	Cas9	Rice	Altered salt-stress response	*Os*RAV2, a transcription factor involved in salt stress response, and its regulatory element. To identify salt-response regulatory element	**Basic research:** Understanding the transcriptional responses and putative functions of the RAV genes and the molecular regulatory mechanisms of plant genes to salt stress	(DUAN et al. 2016)
1	Cas9	Rice	Not relevant	Knock-out of *Os*BEIIb	**Methodology:** To test for induced off-target effects in a closely related paralog (here OsNEIIa), no off-targets found	(BAYSAL et al. 2016)

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1	Cas9	Rice (*Oryza sativa spp. japonica*)	Yield	Overexpressing the brassinosteroid catabolic gene CYP734A4 (common transgenic?) with brd3-D mutant (from transgenic T-DNA insertion library). Knock-out with CRISPR.	**Basic research/R&D:** Cultivation trial in paddy field at China National Rice Research Institute	(QIAN et al. 2017)
1	Cas9	Rice (*Oryza sativa spp. japonica*)	Improved rice blast resistance	Knock-out of the *Os*ERF922 gene (ethylene responsive factors)	**Basic research/R&D:** To develop disease resiatnce	(WANG et al. 2016a)
1	Cas9 multiplexing	Rice (*Oryza sativa spp. japonica*)	Not relevant	21 sgRNAs (up to 10 in one binary vector)	**Methodology:** To develop a new strategy to rapidly construct CRISPR/Cas9-sgRNA system for multiplex editing in plants. Proof of concept for multiplexing	(LIANG et al. 2016)
1	Cas9	Rice & Tobacco	Not relevant e.g. albino phenotype	*Os*PDS (albino), *Os*YSA and *Os*DEP1 genes in rice & *At*BRI1 and *At*PDS3 and *Nt*ALS, *Nt*PDS and *Nt*PDR6 loci in tobacco	**Methodology:** To test a single transcriptional unit (STU) CRISPR/Cas9 system	(TANG et al. 2016b)
1	Cas9 (spCas9)	*Salvia miltiorrhiza* (traditional Chinese med. herb)	Not relevant	Knock-out of the committed diterpene synthase gene (*Sm*CPS1) involved in tanshinone biosynthesis as reporter gene	**Methodology:** Testing GE methodology for *S. miltiorrhiza*	(LI et al. 2017a)

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1	Cas9	Soybean	Nodulation restriction and symbiosis specificity to improve nitrogen fixation	Knock-out of the Glyma.01G165800 and Glyma.01G165800-D genes	**Basic Research:** Functional genetics/reverse genetics to understand genes involved in nodulation	(TANG et al. 2016a)
1	Cas9	Soybean	Albino phenotype	Two soybean phytoene desaturase (PDS) genes, GmPDS11 and GmPDS18 were targeted as visual reporter genes.	**Methodology:** To compare TALENs, CRISPR/Cas9 (with 1: AtU6 promoter and 2: GmU6 promoter)	(DU et al. 2016)
1	Cas9	*Taraxacum kok-saghyz* (rubber producing dandelion)	Increased rubber production	Knock-out of gene for fructan:fructan 1-fructosyltransferase (1-FFT), implicated in inulin biosynthesis was selected as the target, as inulin is an expected antagonist of rubber production	**Basic research/R&D**	(IAFFALDANO et al. 2016)
1	Cas9 (and all variants possible)	Tobacco (*Nicotiana benthamiana*)	For testing transcriptional regulators: nopaline synthase promoter (pNOS) fused to a luciferase reporter	Targeting the endogenous xylosyltransferase (XT) gene	**Methodology:** Developing a modular toolbox (for plant synthetic biology) using tobacco to demonstrate the tool	(VAZQUEZ-VILAR et al. 2016)
1	Cas9	Tobacco (*Nicotiana benthamiana*)	Virus susceptibility (deficiency of antiviral immunity)	Knock-out of AGO2 gene. (plant functional genomics) - reverse genetics	**Basic research**: Study the virus-specific antiviral role of AGO2 Argonaute protein in *N. benthamiana*	(LUDMAN et al. 2017)

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1	Cas9 (transient)	Tobacco (*Nicotiana benthamiana*)	Albino phenotype (white spots)	Phytoene Desaturase (*Nb*PDS) gene	**Methodology:** To establish editing system and show that transient expression works	(MUBARIK et al. 2016)
1	Cas9	Tobacco (*Nicotiana benthamiana*) & Arabidopsis		Creating small deletions by paired nucleases, e.g.: Tobacco: targeting of the immune regulatory genes EDS1 and PAD4 Arabidopsis: Genome editing at a complex *DM2Ler* resistance gene locus; deletion of the tandem EDS1 locus.	**Methodology:** Exploiting multiplexing for induction of inheritable chromosomal deletions of different loci: targeting of small deletions by paired nucleases represents a simple approach for generating mutant alleles segregating as size polymorphisms in subsequent generations, e.g. for use of reverse genetics	(ORDON et al. 2017)
1	Cas9	Tobacco (*Nicotiana tabacum*)	Increased axillary bud growth phenotype	Mutation of NtPIN4 gene to test function ((PIN-FORMED (PIN) auxin efflux proteins)	**Basic research:** characterising PIN gene family in *Nicotiana* species and their function	(XIE et al. 2017)
1	Cas9	Tobacco (*Nicotiana tabacum*)	Not relevant	To show heritability of Cas9 induced mutations. Targeting a GFP gene previously introduced via ag-mediated transformation of leaf segments (with nptII marker gene)	**Basic research:** Showing heritability and fixation of mutations (via haploid technology) in tobacco	(SCHEDEL et al. 2017)

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1	Cas9 (polycistronic tRNA-gRNA)	Tobacco (*Nicotiana tabacum*)	Glycan- engineered plants without beta(1,2)-xylose or alpha(1,3)-fucose	For producing pharmaceuticals (e.g. glycoproteins) in plants, to knock out the two enzymes that are responsible for the addition of plant-specific glycans b(1,2)-xylosyltransferase (XylT) and a(1,3)-fucosyltransferase (FucT) Two XylT genes and four FucT genes (12 alleles) in *N. tabacum* BY-2 suspension cells, using 9 sgRNAs in a polycistronic fashion under one promoter	**R&D:** Develop plant system for the production of proper pharmacological proteins without plant-specific N-glycans with plant-typical residues [b(1,2)-xylose and core a(1,3)-fucose], which can greatly impact the immunogenicity, allergenicity, or activity of the protein	(MERCX et al. 2017) (see also study by HANANIA et al. (2017))
1	Cas9	Tobacco (*Nicotiana tabacum*)	Metabolic engineering for pharmaceutical production	Targeting restriction sites of transgene for fluorescent protein mCherry	**R&D:** Inactivation of proteases and humanization of glycosylation are both important targets which require either gene silencing or gene inactivation	(MERCX et al. 2016)
1	Cas9	Tomato	Flowering irrespective of day length	Knock-out of the florigen paralog and flowering repressor SELF-PRUNING 5G (SP5G)	**Basic research:** To show that loss of day-length-sensitive flowering in tomato can be achieved	(SOYK et al. 2017)

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1	Cas9 (SDN1 deletion)	Tomato	Resistance to powdery mildew	Approach to create homozygous deletions/loss-of-function in *mlo1,* the major contributor to powdery mildew susceptibility	**R&D:** Generating, in less than ten months, Tomelo, a non-transgenic tomato variety resistant to the powdery mildew fungal pathogen	(NEKRASOV et al. 2017)
1	Cas9	Tomato	Facultative parthenocarpy (fertilization-independent fruit set) for heat stress	Knock-out of SlAGAMOUS-LIKE 6 (SlAGL6)	**Basic research:** To enable fruit production under hot/cold temperature and climate stress	(KLAP et al. 2017)
1	Cas9	Tomato	Control of inflorescence architecture of multi-flowered inflorescence	Knock-out mutations of BOP genes (BLADE-ON-PETIOLE), slBOB (interacts with TMF)	**Basic research:** To uncover the underlying mechanisms of the action of the transcription factor gene TERMINATING FLOWER (TMF)	(XU et al. 2016a)
1	Cas9	Tomato	Not relevant	-	**Methodology:** To speed up preparation of binary vector, using one single cloning step for multiple targets, using hairy roots to test functionality of vector	(JACOBS&MARTIN 2016)

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1	Cas9	Tomato (*Solanum lycopersicum*)	Parthenocarpy (seedless fruit without prior fertilisation)	Introduce mutations into *SlIAA9,* a gene controlling parthenocarpy induced by auxin). Many auxin-Induced protein factors (Aux/IAAs), with IAA9 repressing fruit initiation without fertilisation	**Methodology/R&D:** To create rapid system for parthenocarpy in tomato and other horticultural crops, to be independent from pollinators and pollination, especially as climate is not favourable.	(UETA et al. 2017)
1	Cas9	Tomato (*Solanum lycopersicum*)	Albino phenotype/photobleaching;	*Sl*PDS (phytoene desaturase, with albino phenotype in mutant) and *Sl*PIF4 (phytochrome interacting factor, part of a large superfamily of transcription factors)	**Methodology**: Testing CRISPR/Cas in tomatos, esp. for heredity and specificity	(PAN et al. 2016)
1	Cas9	Watermelon *Citrullus lanatus* (Thunb.)	Albino phenotype (as marker trait)	*ClPDS*, phytoene desaturase in watermelon, was selected as the target gene for knock-out because its mutant bears evident albino phenotype.	**Methodology**: Show CRISPR works in watermelon.	(TIAN et al. 2017)
1	CRISPR/Cas9 RNPs (DNA-free system)	Wheat (bread wheat, *Triticum aestivum* L.)	Not relevant	*Ta*GW2 gene: functions in grain weight control, has three homoeologs.	**Methodology:** Improved C/C method for bread wheat. Applicability of RNP based method in wheat	(LIANG et al. 2017a)

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1	Cas9	Wheat (bread wheat, *Triticum aestivu*m L.) also: maize and rice	Not relevant	Target gene: GFP reporter	**Methodology:** To develop and test a replicon-based system for editing of cereal crops using a deconstructed version of the wheat dwarf virus (WDV) – (frequencies 12-fold greater than non-viral delivery).	(GIL-HUMANES et al. 2017b)
1	Cas9	Wheat (Hexa-ploid bread wheat, *Triticum aestivum* L., tetraploid durum wheat, *T. turgi-dum* var. durum)	Modification grain length and weight	Three homoeologues of *Ta*GASR7 (*Ta*GASR7-A1, -B1 and -D1),	**Methodology:** Plants are regenerated from callus cells transiently expressing CRISPR/Cas9 introduced as DNA or RNA.	(ZHANG et al. 2016d)
2	SaCas9 & SpCas9	*Arabidopsis*	Not relevant	-	**Methodology**: To advance multiplex genome editing.	(ZHANG et al. 2017b)
2	SaCas9 & SpCas9	Rice & tobacco	Drooping leaf (rice) and albino phenotype (t) and repressed flowering (t)	DROOPING LEAF (DL) gene for targeted mutagenesis in rice; *Nt*PDS & *Nt*FT4 in Tobacco	**Methodology:** To test *Staphylococcus aureus* Cas9 (SaCas9) in plants	(KAYA et al. 2016)
3	Cas9 SDN-3	Cassava	Glyphosate tolerance	Gene knock-in: Targeted the native cassava EPSPS locus and inserted a 4.2 kb sequence consisting of the 2x35S	**R&D:** Proof of principle study to generate herbicide tolerant cassava	(CHAUHAN et al. 2017)

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				promoter and, coding sequence of the TIPA EPSPS for glyphosate tolerance		
3	Cpf1	Rice	Albino phenotype (as marker trait)	*Os*PDS and *Os*BEL genes	**Methodology:** Testing Cpf1 in rice (as a plant system).	(XU et al. 2017)
3	Cpf1 from *Acidaminococcus* sp. BV3L6 (As) and *Lachnospiraceae* bacterium ND2006 (Lb)	Rice (*Oryza sativa spp. japonica*) and Arabidopsis	Not relevant	Not important what genes targeted. Rice: *Os*PDS, *Os*DEP1 and *Os*ROC5).	**Methodology:** To develop efficient CRISPR/Cpf1 system for plant editing	(TANG et al. 2017)
3	Cpf1 (*Lb*Cpf1 & *As*Cpf1) DNA-free	Soybean & Wild tobacco (*Nicotiana attenuata*)	Increased oleic acid (soybean)	To show method works for modification of FAD2 paralogues (FAD2-1A, FAD2-1B) in soybean and AOC (ALLEN OXIDE CYCLASE gene) in wild tobacco	**Methodology/R&D:** To show Cpf1 is a tool for DNA-free editing of plant genomes to modify desired nutritional trait, e.g. oleic acid level in soybean oil	(KIM et al. 2017b)

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4	Cas9 & Cpf1	Rice	Not relevant (Eightfold) reduction in stomatal density as marker	Early developmental gene EPFL9 orthologue in rice - (Epidermal Patterning Factor Like-9) as marker to test the efficiency of CRISPR/Cas9 and CRISPR/LbCpf1 in rice	**Methodology, proof of concept:** To develop a tool for functional study of early developmental genes through the generation of knock-outs in plants	(YIN et al. 2017)
5	Cas9 & ssODNS SDN2	Flax (*Linum usitatissimum*)	Herbicide resistance (HR) Conversion BFP to GFP	To edit HR (EPSPS) genes and a stably integrated blue fluorescent protein gene (BFP) into a GFP gene	**Methodology:** To test SDN2 approach also using single stranded Oligonucleotides (ssODNs)	(SAUER et al. 2016b)
5	Cas9 gRNA RNP SDN1 & SDN2	Maize	Herbicide resistance	Four genomic regions, liguleless1 (LIG), acetolactate synthase (ALS2) and two male fertility genes (MS26 and MS45), were targeted by purified Cas9 protein	**Methodology/R&D:** To introduce specific ALS herbicide resistance	(SVITASHEV et al. 2016)
5	Cas9 SDN2 (template supplied on binary vector) similar approach used with TALEN	Potato (*Solanum tuberosum* L.)	Herbicide tolerance	Specific modification of acetolate synthase 1 (ALS1).	**Methodology:** To devise a system that allows for specific modification via HDR, utilising replicons from geminivirus	(BUTLER et al. 2016)
5	Cas9 for HDR SDN2	Rice	Herbicide resistance	Altering ALS1 gene (Acetolactate Synthase 1).	**Methodology**: For developing HDR in rice	(SUN et al. 2016b)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
5	Cas9 (NHEJ & HR)	Tomato (*S. lycopersicum* and wild typ *Solanum pimpinellifoliu m*)	Fruit colour (bicolour, yellow and yellow with red spots fruits)	Using Phytoene synthase 1 (PSY1) gene as 'visual marker'; to test potential targeted recombination between homologous chromosomes in plants	**Methodology/basic research:** To induce HR between homologous chromosomes in response to targeted DSBs at a specific genomic location	(HAYUT et al. 2017)
6	Cas9 SDN3	*Arabidopsis thaliana*	GFP reporter gene	Homology-directed repair gave rise to the eGFP sequence, replacing the local deleted *AtTFL1* region (Terminal Flower)	**Methodology:** To apply new strategy for gene replacement: deletion/replacement system	(ZHAO et al. 2016)
6	Cas9 as SDN3 (HR homology-directed DNA repair)	Maize	Higher yield under drought (reduced ethylene sensitivity)	To insert or swap the endogenous GOS2 promoter for moderate level of constitutive expression. Tested in field trials	**R&D**: To improve maize grain yield under field drought stress conditions. To use CRISPR to create overexpressing variants of ARGO58, which is known to improve grain yield under drought conditions	(SHI et al. 2017a)
6	Cas9 SDN1: mostly deletions SDN3: (by TGR & TGI)	Moss: *Physcomitrell a patens*	Resistance to 2-fluoroadenine Resistance to antibiotics	Knock-out of endogenous reporter gene, *Pp*APT (adenine phosphoribosyltransferase), (confers resistance to 2-fluoroadenine) Insertion of circular donor plasmid with an antibiotic resistance (G418-resistant	**Methodology:** First successful use of genome editing in a nonvascular plant **Basic research**: HDR in moss: illegitimate recombination; *Pp*RAD51 protein only partially needed for HR	(COLLONNIER et al. 2017)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
				gene) gene flanked by DNA fragments homologous to the genomic regions flanking the target	SDN3 by targeted gene replacement (TGR) or by targeted gene insertion (TGI)	
6	Cas9 SDN2/3	Rice	Glyphosate resistance	Mutated EPSPS gene (by gene replacement)	**Methodology** and proof of principle.	(LI et al. 2016a)
7	nCas9 (nickase) for adjacent genomic SSBs	*Arabidopsis thaliana*			**Basic research**: Tandem-sequence duplication (genome evolution aspect).	(SCHIML et al. 2016)
7	nCas9 (nickase) with paired gRNAs	Rice (*Oryza sativa* L. cv. Nipponbare)	Not relevant	Targeted rice *Os*DMC1A gene as the 'on-target' gene, and the *Os*DMC1B gene, a paralog of *Os*DMC1A as the 'off-target' gene.	**Methodology**: To analyze on- and off-target mutation frequency in rice calli and regenerated plants using Cas9 nuclease or Cas9 nickase with paired gRNAs	(MIKAMI et al. 2016)
8	nCas9 (nickase) cytidine deaminase	Rice	Semi dwarf growth (also intended: increased nitro-gen fixation, however no results reported in this case)	To develop a base editing system for plants (C to T) using rat APOBEC1 (cytidine deaminase) [APOBEC1-XTEN-Cas9 (D10A) fusionprotein] – testing on a DELLA protein (SLR1) and NRT1.1B, a nitrogen transporter	**Methodology:** Base editing of important agricultural traits	(LU&ZHU 2017)

Cat	Nuclease variant	Crop Plant	Conferred Trait/ specific Characteristic of nGM plant	Purpose of the application, Approach, Target Gene(s)	Purpose of the Study	Reference
8	BE3 (Base Editor, 3rd generation) (nCas9, cytidine deaminase, and UGI)	Rice	Not relevant	Three targets: one in *Os*PDS, which encodes a phytoene desaturate, and two in *Os*SBEIIb, a starch branching enzyme IIb in rice	**Methodology:** To test ability to introduce precise point mutations G to A in target sequence and to test efficacy of desired base editing vector	(LI et al. 2017b)
8	Cas9 & nCas9 cytidine deaminase & dCas9 uracil glycosylase inhibitor	Rice Wheat Maize	Reporter gene with modified fluorescence (Blue to green color). & others	To achieve targeted "base editing": BFP to GFP and knock-out of three endogenous genes in rice (*OsCDC48*, *OsNRT1.1B, OsSPL14*), *TaLOX2* in wheat and *ZmCENH3* in maize	**Methodology work** T test and describe the optimization of base editing in cereal crops using a n/dCas9 plant base editor	(ZONG et al. 2017)
8	Fusion of CRISPR(d/n)/Cas9 and activation-induced cytidine deaminase (Target-AID)	Rice (*Oryza sativa*) Tomato (*Solanum lycopersicum*)	Rice: herbicide tolerance (hygromycin and imidazolinon)	Rice first: EGFP as a reporter assay to establish base editing efficiency *in planta*. Multiplex: FTIP1e gene (2 sgRNAs, hygromycin) & gene for acetolactate synthase (ALS).	**Methodology:** Nuclease-deficient Cas9 (dCas9) or nickase CRISPR/Cas9 (nCas9) fused to *Petromyzon marinus* cytidine deaminase (PmCDA1)1 and sgRNAs to introduce point mutations (base editing).	(SHIMATANI et al. 2017)

7.2 Annex 2: Applications of TALE-Nuclease systems for genome editing (TALEN)

Table 8: TALEN applications retrieved from scientific literature (2011-2017)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of nGM plant	Purpose of the Application, Approach, Target Gene(s)	Purpose of the Study	Reference
SDN1	*Arabidopsis thaliana*		Small, germ line-transmitted deletions, CLV3 coding sequence	**Methodology/Basic research**: CLV3 locus as a test case for the development of efficient and specific genome editing strategies	FORNER et al. (2015)
SDN1	*Nicotiana benthamiana*	Altered glycosylation pattern	Knock-out of two (1,3)-fuco-syltransferase (FucT) and two (1,2)-xylosyl-transferase (XylT) genes	**Basic research:** To improve capacity to produce glycoproteins	LI et al. (2016b)
SDN3	Tomato	Purple plant tissue	Anthocyanin mutant1 (ANT1)	**Methodology/Basic research**	Cermak et al. (2015) IN SUN et al. (2016a)
SDN3	Potato	Herbicide resistance	ALS with mutant site (geminivirus-based replicons)	**Methodology/R&D:** Use of geminivirus expression system to induce HR	Butler et al. (2016) IN SUN et al. (2016a)
SDN1	Potato	Improving cold storage and processing traits	Knock-out of the vacuolar invertase gene (VInv)	**Basic research/ R&D**	CLASEN et al. (2016)
SDN3	Potato	Not relevant	DNA insertion	**Methodology**	FORSYTH et al. (2016)
SDN1	Potato	Herbicide resistance	Transient TALEN expression in protoplasts, ALS gene	**Methodology/R&D**	NICOLIA et al. (2015b)
SDN1	Soybean	Establish varieties low in polyunsaturated fatty acids	Targeted mutagenesis of the fatty acid desaturase 2 gene family (FAD2-1A and FAD2-1B)	**R&D:** To develop a soybean producing high oleic acid oil (Calyno premium cooking oil)	HAUN et al. (2014)
SDN1	Soybean	Altered composition	Knock-out of the FAD3 linoleate desaturase genes	**R&D:** TALENs targeting FAD3 were directly delivered to fad2-1a and fad2-1b knockout lines to produce triple knockout fad2-1a fad2-1b fad3 plants	DEMOREST et al. (2016)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of nGM plant	Purpose of the Application, Approach, Target Gene(s)	Purpose of the Study	Reference
SDN3	Barley	Not relevant	GFP (transgene)	**Methodology:** Marker gene expression	Budhagatapalli et al. (2015) IN SUN et al. (2016a)
SDN1	Barley	Not relevant	Gene knock-out, gfp-specific TALEN sequence	**Basic research:** Description of loss-of-function	GURUSHIDZE et al. (2014)
SDN1	Barley	Composition (Phytate storage)	Design and assembly of three candidate TALENs specific for the same target region in the promoter of the PAPhy_a gene	**Methodology:** Report of the assembly of several TALENs for a specific genomic locus in barley	WENDT et al. (2013)
SDN1/SDN3	Wheat	Resistance to powdery mildew	Simultaneous editing of three homeooalleles that encode mildew resistance locus (mlo)	**Basic research**: TALEN-induced mutation of all three TaMLO homoeologs confers broad-spectrum resistance to powdery mildew	WANG et al. (2014a)
SDN1	Maize	Phosphorus metabolism (Phytic acid biosynthesis)	pds, ipk1a, ipk, mrp4	**Basic research/R&D**	Liang et al. (2014) IN SOVOVÁ et al. (2017)
SDN1	Maize	Glossy phenotype	Generation of heritable mutations at the glossy2 (gl2) locus	**Basic research**	CHAR et al. (2015)
SDN1	Rice	Disease resistance	Rice bacterial blight susceptibility gene Os11N3	**Basic research/R&D**	LI et al. (2012)
SDN3	Rice	Herbicide resistance	ALS with mutant site	**R&D**	Li et al. (2016) IN SUN et al. (2016a)
SDN1	Rice	Grain quality and rice architecture	dep1, ckx2, badh2, sd1 genes	**Basic research/R&D**	Shan et al. (2013a) IN SOVOVÁ et al. (2017)
SDN1	Rice	Composition (increased fragrance)	Targeted knock-out of the OsBADH2 gene	**Basic research/R&D**: Creation of fragrant rice	SHAN et al. (2015)
SDN1	Rice	Enhances the Storage Tolerance	Mutagenesis of Lipoxygenase LOX3	**Basic research/R&D**: To develop rice with improved seed quality and shelf life	MA et al. (2015)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of nGM plant	Purpose of the Application, Approach, Target Gene(s)	Purpose of the Study	Reference
SDN2	Rice	Herbicide resistance (Glyphosate)	Co-transformation of TALEN and chimeric RNA/DNA oligonucleotides on the rice OsEPSPS gene	**Methodology/R&D**: Improved editing efficiency and induction of herbicide resistance	WANG et al. (2015)
SDN1	Rice	Resistance to *Xanthomonas oryzae*	Disruption of OsSWEET14 (Os11N3) promoter region	**Basic research/R&D**: To develop rice with increased disease resistanc	Li, T. (2012) IN WRIGHT et al. (2014)
SDN1	Rice	Knock-out of various rice genes of agronomic value (male-sterility, increased grain yield, drought resistance)	OsCSA, OsPMS3, OsDERF1, OsGN1a, OsTAD1, OsMST7 and OsMST8, genes	**Methodology**: Study of the efficiency of the TALEN system in rice as well as the nature and inheritability of TALEN-induced mutations	ZHANG et al. (2016b)
SDN1	Sugarcane	Reduction lignin, altered content for production of bioethanol	Targeted mutagenesis in a highly conserved region of the caffeic acid O-methyltransferase (COMT)	**Basic research/R&D**: To develop feedstock for biofuel production	JUNG&ALTPETER (2016)
SDN1	*Brassica oleracea*	High bolting resistance	TALEN-based disruption of vernalization determinant allele FRIGIDA (FRI)	**Basic research**: Proof of principle	SUN et al. (2013)

7.3 Annex 3: Applications of ZFN systems for genome editing

Table 9: ZFN applications retrieved from scientific literature (2011-2017)

SDN	**Crop Plant**	**Conferred Trait/spec. Characteristic of the nGM plant**	**Target Gene/ Integrated Gene**	**Specifics of Method, Approach, Gene, Construct**	**Purpose of the Study**	**Reference**
SDN3	Tobacco (applicable to other plants)	Herbicide toler-ance (bialophos, glufosinate), if promoter is integrated.	PAT (used as reporter gene)	Transformation with genome editing target construct, then with ZFN expression construct and donor DNA	**Methodology**: Detailed description of materials and methods for transgene integration	(STRANGE& PETOLINO 2012)
SDN1	Apple (*Malus domestica*) & Fig (*Ficus carica*)	GUS expression (as reporter in colour assay)	Transgene with mutated uidA gene (Beta-glucuronidase or GUS)	ZFN constructs with 35S (transient) or heat-shock (stable transformation) promoter. Aim is the deletion of stop codon in the mutated target reporter gene and to compare the two approaches	**Methodology**: To show that ZFN mediated genome editing (knock-out of endogenous genes) is feasible in perennial fruit trees	(PEER et al. 2015)
n/a	Arabidopsis	Not relevant	-	-	**Methodology**: To design and construct multi-transgene binary vectors utilizing ZFNs and homing endonucleases	(ZEEVI et al. 2012)
SDN1	Arabidopsis	Not relevant	Not relevant	Detailed description of method protocol to introduce mutations in Arabidopsis using tailor-made ZFNs	**Methodology:** Development of alternative techniques to knock out target genes).	(QI et al. 2014)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
SDN1	Arabidopsis	n/a Approach aims to create genomic deletions. (Gene cluster deletions ranged from a few kb to 55 kb.)	Gene clusters (e.g. RPP4) and tandemly arrayed genes (TAGs).	ZFN pairs produced for cutting multiple sites within gene clusters or TAGs	**Methodology/Basic research:** Exploring method aiding reverse genetics that will allow for targeted chromosomal rearrangement and to delete multiple gene copies or to trigger genomic rearrangements.	(QI et al. 2013b)
SDN1	Arabidopsis	GUS expression (as reporter in colour assay)	Non-functional mutated GUS reporter gene	Approach to develop a method for egg-specific ZFN expression by utilising.Testing ZFN activity and with blue stain visualisation at seed stages (repair of non-functional GUS). Expressing RAD54 enhances ZFN efficiency	**Methodology**: To develop and test system for introducing targeted mutations specifically in egg cells after introduction of ZFN constructs with floral dip method	(EVEN-FAITELSON et al. 2011)
SDN1	Arabidopsis	Not relevant	-	Description of detailed protocol for making targeted mutations in Arabidopsis with ZFN expression induced during germination	**Methodology**: To develop a method for ZFN expression during germination of Arabidopsis	(ZHANG&VOYTAS 2011)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
SDN1 & SDN3	Arabidopsis	Different endogenous genes used as targets and as reporter system (PCR and sequence analysis)	ADH1, TT4, MPK8	To test the effect of specific repair pathway proteins on the efficiency of ZFNs (using previously constructed mutant lines lacking the repair proteins *ku70, lig4, smc6b)*.	**Basic research/Methodology:** To enhance the frequency of NHEJ & HDR by manipulating DNA repair pathways Both NHEJ and HDR were tested and analysed for method efficiency and size of indels	(QI et al. 2013a)
SDN2	Arabidopsis	Herbicide resistance (to butafenacil-herbicides)	Endogenous protoporphyrinogen oxidase (PPO) gene	Genome Editing approach targeting PPO gene, using 'repair T-DNA' to introduce two changes establishing herbicide tolerance.	**Methodology**: To show ZFNs can correctly repair or alter endogenous genes in Arabidopsis via HDR.	(DE PATER et al. 2013)
SDN1 & SDN3	Arabidopsis (mutants)	Not relevant	ADH1 (as model/target)	ZFN expression plasmid, and/or donor plasmid. Using Arabidopsis mutant lines for genes involved in DNA repair: H2AX and small RNA pathway genes.	**Basic research**: To investigate the role of genes involved in DNA repair and contribution to HR and/or NHEJ, in particular looking at the role of diRNAs (DSB-induced small RNAs) and the two H2AX isoforms.	(QI et al. 2016b)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
SDN2/3	Maize	Herbicide resistance (as marker) and loss of yellow fluorescence (reporter gene).	Transgenic target gene and donor gene	Crossing a GM ZFN plant with a GM plant containing two separate, unlinked transgenes, but with areas of homology, ZFN target sites and TLPs. (Transgene 1: target construct with a YFP reporter gene and a trait gene. Transgene 2: donor construct, harboring a promoterless glyphosate tolerance gene). ZFN activity results in replacing the YFP coding sequence with the herbicide tolerance coding sequence.	**Methodology/R&D**: attempting to create a trait stacking system via intra-genomic homologous recombination, employing 'trait landing pads' (TLPs). Stacking results in a 'molecular stack', thereby preventing segregation of stacked transgenes.	(KUMAR et al. 2016)
SDN3	Maize	Glufosinate herbicide tolerance (used as marker). Lost trait due to replacement: Yellow fluorescent protein (YFP) as reporter.	Transgenic target construct (YFP reporter gene). YFP coding sequence is replaced with the PAT-3' UTR coding sequence from donor construct	Employing a unique intron between promoter and the coding sequence of the YFP reporter gene, with the intron serving as homology target integration of donor construct, containing a promotorless PAT HR marker gene by HDR	**Methodology/R&D**: Proof of principle study to enable easy and sequential transgene stacking into one locus, to prevent segregation of transgenes (modular gene targeting system for transgene stacking simultaneously exchanging a selectable marker while integrating additional transgenes)	(KUMAR et al. 2015)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
SDN3	Maize	Stacked herbicide tolerance	aad1 herbicide tolerance gene (aryloxyalkanoate dioxygenase) introduced by SDN3 approach using the TLP (trait landing pad) of pat gene (phosphinothricin acetyltransferase) construct as target locus.	The first TLP, together with the PAT herbicide tolerance gene, was randomly inserted into the genome to provide target sequence for subsequent SDN3-mediated transgene integration.	Methodology/R&D: To develop a method for easy trait (transgene) stacking via HDR, to ensure co-segregation of traits. Aimed to create modular 'trait landing pads' (TLPs) for subsequent targeted transgene integration and trait stacking in crop plants.	(AINLEY et al. 2013)
SDN1	Poplar	Attempted trait sterility (no success reported)	LEAFY and AGAMOUS (PtAG1, PtAG2 and PtLFY)	Use of heat-inducible ZFN system. The targeted transcription factors are key regulators for floral initiation and floral organ identity.	**Methodology/R&D:** To establish floral sterility by knocking out of relevant genes (This trait might enable the commercial use of transgenic trees)	(LU et al. 2016)
SDN1	Rice	No specific trait. (indels of different sizes at target site)	Previously integrated CCR5 target site	Approach to investigate the size of indels at a locus for site-specific integration to compare ZF-nuclease with I-SceI nuclease for accuracy, both with constitutive as well as heat-shock induced expression of the nucleases, using a Cre-lox system for targeted transgene integration with different site-directed nucleases (ZFN and I-Sce I)	**Methodology/R&D:** Proof of concept study towards gene stacking through multiple rounds of transformation and marker gene removal using a combination of recombinases and nucleases.	(NANDY et al. 2015)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
SDN1	Rice (var. *indica*)	Established trait after repair with ZFN: expression (as reporter in colour assay)	Non-functional, mutated GUS reporter gene with stop (TGA) codon	A) Testing of 3 different ZFN constructs for transformation efficiency. B) For ZFN-mediated repair of mutated GUS gene, GUS activity was used to screen for desired outcome	**Methodology**: To establish and test a method to experimentally identify 'safe harbor loci' for the integration of transgenes. Such loci must be in non-coding regions and possess high gene expression can be used for subsequent R&D projects.	(CANTOS et al. 2014)
SDN1	Soybean	Not relevant	-	Design of easy and rapid method to engineer ZFNs for the purpose of inserting targeted mutations into polyploid or paleopolyploid genomes that have two or more copies for most genes, like soybean	**Methodology**: To devise an improved method for ZFN construction	(CURTIN et al. 2013)
SDN1	Soybean	Double mutant: reduced seed size, aborted seedling development.	DICER-LIKE1 (DCL1) homologs, DCL1a and DCL1b	Creating single and double mutants (dcl1a/dcl1b)	**Basic research**: Functional genomics to confirm the functional role of DCL1a and DCL1b in the soybean miRNA pathway.	(CURTIN et al. 2016)
SDN1	Soybean (*Glycine max*)		DICER-LIKE (DCL), RNA- DEPENDENT RNA POLYMERASE (RDR), and HUA ENHANCER1 (HEN1) family members	Devise ZFNs that recognise duplicate gene copies. Utilise hairy root system to test efficiency of ZFNs prior to full plant transformation.	**Methodology/Basic research**: ZFNs as tool for plant functional genomics (studying the functions of genes involved in RNAi mediated silencing	(CURTIN et al. 2011)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
SDN3	Tobacco (BY-2 cell suspension cultures)	RFP (red fluorescent protein), kanamycin resistance and bialaphos herbicide resistance	Integrated transgenic target construct (AHAS, GFP and 3' portion of DSM2 marker gene)/donor construct (RFP, nptII and 5' portion of DSM2 marker gene)	Sequence replacement: in target construct by donor construct, both previously inserted via *Agrobacterium*-mediated transformation; both constructs designed with corresponding homology areas	**Methodology/R&D**: To devise and test a method for gene replacement or multigene exchange that could also be applied in a context of commercial development	(SCHNEIDER et al. 2016)
SDN2 & SDN1	Tobacco (*Nicotiana tabacum*)	GUS expression (as reporter in colour assay), and antibiotic resistance	Integrated non-functional translational fusion *gus:nptII* reporter gene with a 600bp deletion	T-DNA construct harbouring geminivirus-based replicons (GVRs) containing sequences for ZFN as well as a 600bp repair template for SDN2 experiment	**Methodology**: to devise a system for gene targeting with enhanced frequency, utilising GVRs (geminivirus-based replicons) for transient expression of SDNs and the delivery of templates	(BALTES et al. 2014)
SDN1 approach for gene replacement via NHEJ	Tobacco (*Nicotiana tabacum*) & Arabidopsis	Herbicide resistance (hygromycin herbicides)	Target: green fluorescent protein (GFP) transgene; Donor: promoterless hygromycin B phosphotransferase (htp) construct without sequence homology to target sequences	To transform plants a) with 'acceptor DNA' with ZFN target site flanking the coding sequence of the GFP reporter gene, and subsequently b) with ZFN construct and donor DNA, containing a promotorless gene flanking ZFN recognition sites	**Methodology**: To show NHEJ can be utilized for transgene replacement and gene stacking in plants.	(WEINTHAL et al. 2013)
SDN1 approach to generate deletions	Tomato	Timing of flower and fruit setting, fruit and seed morphology.	LEAFY-COTYLEDON1-LIKE4 (L1L4)	Combining targeted deletions and metabolic screens to identify genes which are involved in and required for expression of a	**Basic research/R&D**: Studying major regulatory transcription factors relevant to facilitate accelerated breeding	(GAGO et al. 2017)

SDN	Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Target Gene/ Integrated Gene	Specifics of Method, Approach, Gene, Construct	Purpose of the Study	Reference
				particular trait		
SDN1	Tomato	Multitude of altered characteristics and phenotypes throughout development. (incl. sterility, early flowering, more flowers per inflorescence, height, leaf shape, fruit shape)	LEAFY-COTYLEDON1-LIKE4 (L1L4), the beta subunit of transcription factor NF-Y	Major regulatory transcription factor	**Methodology/basic research**: To devise a new ZFN platform with DNA binding sites 1kb apart. For functional genomics and to introduce mutational pleiotropy at the L1L4 locus.	(HILIOTI et al. 2016)

7.4 Annex 4: Applications of Meganuclease Systems for genome editing (MN)

Table 10: MN applications retrieved from scientific literature (2011-2017)

SDN	Crop Plant	Conferred Trait/ spec. Characteristic of the nGM plant	Purpose of the Application, Approach, Target Gene(s)	Purpose of the Study	Reference
SDN1/2/3	Arabidopsis	Not relevant	To establish customised homing endonuclease PB1	**Methodology**: Development and test of a synthetic homing endonuclease, PB1, derived from the I-CreI endonuclease of *Chlamydomonas reinhardtii*	ANTUNES et al. (2012)
SDN1	Maize	Male sterility	Gene knock-out liguless 1	**Methodology/ Basic research**: Development of methods to target specific endogenous genes involved in developmental regulation	Gao et al. (2010) and Lyznik et al. (2012) In (DABOUSSI et al. 2015)
SDN3	Cotton	Herbicide tolerance	Insertion of additional trait genes (hppd, epsps)	**R&D**: Targeted molecular trait stacking in cotton through targeted double-strand break induction	D'HALLUIN et al. (2013)
SDN3	Barley	Not relevant	Gene replacement by targeted double-strand break induction	**Methodology/Basic research**: Analysis of gene targeting in barley using a model system based on double-strand break (DSB) induction by the meganuclease I-*Sce*I and a transgenic, artificial target locus	WATANABE et al. (2016)
SDN1	Maize	Male sterility	Gene knock-out ms26 gene	**R&D**: To establish knock-out alleles of endogenous genes to develop lines with male sterility phenotypes	Djukanovic et al. (2013) In: KAMTHAN et al. (2016) and SOVOVÁ et al. (2017)

7.5 Annex 5: Applications of ODM for genome editing

Table 11: ODM applications retrieved from scientific literature (2011-2017)

Crop Plant	**Conferred Trait/spec. Characteristic of the nGM plant**	**Purpose of the Application, Approach, Target Gene(s)**	**Purpose of the Study**	**Reference**
Oilseed rape (*Brassica napus*)	Herbicide resistance	Generation of targeted sequence modifications in the acetolactate synthase (ALS) gene by ODM	**R&D**: end-protected single-strand oligonucleotides were used to generate sequence conversions in the targeted ALS gene	(GOCAL et al. 2015)
Flax (*Linum usitatissimum*)	Herbicide resistance	Generation of Glyphosate-resistant plants by ODM-based modification of the EPSPS gene	**Methodology/R&D**: Development of a combined approach for high-frequency genome editing using oligonucleotide templates in conjunction with site directed nucleases introducing DSBs near the site of editing	(SAUER et al. 2016b)

7.6 Annex 6: Applications of RdDM

Table 12: RdDM applications retrieved from scientific literature (2011-2017)

(VITGS: virus-induced transcriptional gene silencing; TGS: transcriptional gene silencing; PTGS: post-transcriptional gene silencing; hpRNA: hairpin RNA; dsRNA: double-stranded RNA; siRNA: short inhibitory RNA; GFP: green fluorescent protein; S0, S1, S2: silenced generation 0, 1, 2, n/a: not applicable)

Method of RdDM	**Crop Plant**	**Conferred Trait/ spec. Phenotype nGM plant**	**Target Gene**	**Trigger**	**Stability of effect in the absence of trigger?**	**Epigenetic Effect**	**Modification, Target Gene(s), gene constructs, approach**	**Purpose of the Study**	**Reference**
Transgene-mediated	Arabidopsis	Clustered stomata on cotyledons	Endogenous TMM gene (Too Many Mouths), as a model target gene (loss-of-function marker)	Single stranded RNA (sense and antisense) and IR RNA	No	Transcriptional gene silencing (TGS) by methylation and histone modification	Separately targeted four Arabidopsis endogenes: TMM, FHY1, HFR1 and PHYB using floral dip transformation.	**Methodology/Basic research**: Investigating different strategies for gene silencing of endogenes and to show that single stranded RNA is sufficient to induce TGS, in particular antisense to promoter sequence.	DENG et al. (2014)
Transgene-mediated	Maize	Male sterility	MS45 (male fertility) (strictosidine synthase) genes	pIR RNAs (promoter-inverted repeats RNA)	n/a	Methylation of promoter	Using transgenic anther specific promoters from rice and Arabidopsis to express the maize MS45 gene in an ms45/ms45 male sterile background	**Methodology/R&D**: (1) to devise a genetic system for male sterility for seed production, (2) to study influence of sequence similarity between pIR RNA and target promoter.	CIGAN et al. (2014)

Method of RdDM	Crop Plant	Conferred Trait/ spec. Phenotype nGM plant	Target Gene	Trigger	Stability of effect in the absence of trigger?	Epigenetic Effect	Modification, Target Gene(s), gene constructs, approach	Purpose of the Study	Reference
VITGS (CMV-A1)	Petunia (P), Tomato (T)	P: change in flower colouring (20% have wider white area); low pollen germination. T: inhibition of fruit ripening	Endogenous CHS-A gene (chalcone synthase) - Petunia & endogenous LeSPL-CNR gene (colourless non-ripening) - Tobacco	Viral produced dsRNAs	P: S1 (only few survived) T: S1 (histone modification retained, though now mottled fruit)	Both plant species: promoter methylation and histone modification	Studying the virus b2 protein (silencing suppressor, ferrier of siRNA to nucleus) with visual traits.	**Methodology**: Proof of principle to show that heritable gene silencing can be induced by targeting dsRNA to endogenous promoters, using Cucumber Mosaic Virus (CMV) vector.	KANAZAWA et al. (2011a)
VITGS (CMV-A1)	Petunia; Arabidopsis	Partial male sterility, seed coat colour change	Endogenous CHS-A gene (chalcone synthase)	Viral produced dsRNAs	S1 (as detailed in previous paper:)	Methylation of promoter - strong but not complete silencing.	Comparator of the study is a co-suppression line with CHS-A endogenous gene and homologous transgene, which gives rise to PTGS.	**Methodology/ R&D**: To compare TGS effect to co-suppression.	KANAZAWA et al. (2011b)
VITGS (ALSV)	Petunia & Tobacco (*N. benthamiana*)	Strongly reduced expression of reporter gene in tobacco & white petals instead of red ones in petunia	35S promoter of transgenic reporter gene (GFP gene) (tobacco) and chalcone synthase-A (CHS-A) (petunia)	Promoter sequences as siRNA	S2-S4 for reporter gene in tobacco.	Methylation of promoter – strong silencing.	Using apple latent spherical virus (ALSV) as silencing vector.	**Methodology/Basic research**: To test an *Agrobacterium*-mediated inoculation system for the ALSV vector and test if ALSV-VITGS can induce DNA methylation in an endogenous plant gene	KON&YOSHIKAWA (2014)

Method of RdDM	**Crop Plant**	**Conferred Trait/ spec. Phenotype nGM plant**	**Target Gene**	**Trigger**	**Stability of effect in the absence of trigger?**	**Epigenetic Effect**	**Modification, Target Gene(s), gene constructs, approach**	**Purpose of the Study**	**Reference**
(1) transgene for pIR expression (2) hetero-Transgrafting with GM Tobacco scion - mobile siRNA	Potato (*Solanum tuberosum*)	(a) Reduced expression of reporter gene construct & (b) A waxy potato with reduced amylose starch content	35S promoter of transgenic reporter gene (GFP gene) & Granule-bound starch synthase I (GBSSI) endogenous gene	siRNA resulting from IR RNA of promoter sequence	Silencing of transgene passed on to next progeny of tubers (reduced expression); Silencing of endogenous GBSSI gene not passed to next generation in current study	TGS: Methylation of promoter	Transgenic potato with 35S. GFP reporter gene was used as rootstock, with GM tobacco scion as siRNA donor for 35S promoter silencing. Wild type potato as rootstock with GM tobacco scion as siRNA donor for endogenous GBSSI promoter sequences	**R&D**: To show hetero-transgrafting will result in TGS that can be used for breeding purposes of vegetativly propagated crops.	KASAI et al. (2016a)
Agroinfiltration of leaves & transgrafting – mobile hpRNA	Tobacco (*Nicotiana benthamiana*)	Reduced expression of reporter gene construct	Transgenic 35S:GFP reporter gene	hpRNA (hairpin) expressed in GM rootstock or scion as hpRNA donor	Yes. TGS maintained throughout in vitro regeneration and passed on to the selfed progeny	Methylation of 35S promoter. (weak to strong silencing, depending on tissue and donor graft)	Applying agroinfiltraton of leaves, which then are removed (1-13 days) grafted onto siRNA rootstock or with siRNA scion	**Methodology/R&D**: To test if siRNA that induces TGS in an agroinfiltration system is graft transmissible and inherited by progeny	BAI et al. (2011)

7.7 Annex 7: Applications of Cisgenesis

Table 13: Cisgenesis applications retrieved from scientific literature (2011-2017)

Crop Plant	**Conferred Trait/spec. Characteristic of the nGM plant**	**Purpose of the Application, Approach, Target Gene(s)**	**Purpose of the Study**	**Reference**
Apple	Scab resistance	Cisgenic apples expressing the *Rvi6* scab resistance gene	**R&D:** Molecular characterization of three cisgenic lines of apple 'Gala' carrying the Rvi6 scab resistance gene (data required for risk assessment acc. to Regulation (EC) 1829/2003)	VANBLAERE et al. (2014)
Grape vine	-	Heat-inducible promotor activates a recombinase which removes the selectable marker *nptII*, then a reporter gene *gus* is expressed	**Methodology**: Test of heat-shock inducible system for marker gene removal Proof of concept study	DALLA COSTA et al. (2016)
Grape vine	-	Improvement of important traits in grape vine	**Basic research**: Identification of grape vine promotors and characterization of their expression profiles	ESPINOZA et al. (2013)
Potato	Late blight resistance	Marker-free cisgenic potato[10] with resistance genes from *S. stoloniferum* and *S. venturii* (*Rpi-sto1* and *Rpi-vtn1.1*)	**R&D**: Development of marker-free cisgenic potato plants resistant to late blight	JO et al. (2014)[11]
Potato	Late blight resistance	Durable late blight resistance in potato cultivars by stacking of R genes derived from crossable wild potato species	**R&D**: Summary of all sub-projects concerning risk assessment conducted in the course of the "Durable Resistance in Potato against Phytophthora (DuRPh)" research project	HAVERKORT et al. (2016)
Chinese cabbage	-	Application of the ALS gene as a cisgenic selectable marker	**R&D**: Development of a cisgenic selectable marker for *Agrobacterium*-mediated transformation in Chinese cabbage	KONAGAYA et al. (2013)
Banana	Increased pro-vitamin A carotenoids (pVAC) content	Banana cultivars with increased pro-vitamin A carotenoids by expression of PSY genes with higher enzymatic activity	**Basic research**: Isolation and functional characterisation of banana *phytoene synthase (PSY)* genes as potential cisgenes	MLALAZI et al. (2012)

[10] PCR war used for the selection of transformed plants
[11] cited in Cardi 2016

Crop Plant	Conferred Trait/spec. Characteristic of the nGM plant	Purpose of the Application, Approach, Target Gene(s)	Purpose of the Study	Reference
Banana	Fusarium wilt resistance	Banana cultivars with resistance to fusarium wilt and tolerance to abiotic stress	**Basic research**: Review of scientific knowledge on plant-pathogen interaction (*Fusarium oxysporum*) and candidate R genes identified in various crop plants and wild banana species	SWARUPA et al. (2014)
Barley	Improved grain phytase activity	Overexpression of HvPAPhy_a gene	**R&D**: Development of feedstock with improved nutritional quality	Holme et al. (2012) IN: KAMTHAN et al. (2016)

7.8 Annex 8: Applications of Intragenesis

Table 14: Intragenesis applications retrieved from scientific literature (2011-2017)

Crop Plant	**Conferred Trait/spec. Characteristic of the nGM plant**	**Purpose of the Application, Approach, Target Gene(s)**	**Purpose of the Study (**	**Reference**
Sugarcane	Resistance to ALS-inhibiting herbicides	Use of a mutant sorghum acetolactate synthase gene (mALS) as intragenic selectable marker (additional genetic elements derived from Sorghum)	**Methodology**: Development of an intragenic gene transfer and selection protocol for sugarcane	DERMAWAN et al. (2016)
Strawberry	Resistance to glyphosate	Intragenic vectors in strawberry	**Basic research/R&D**: Assessment of various promotors (constitutive and fruit-specific) and a selectable marker (overexpression of the native FvEPSPS gene) for the development of intragenic vectors for strawberries	CARVALHO&FOLTA (2017)
Citrus	-	Intragenic vector in *Citrus* spp.	**R&D**: Development of an intragenic vector for applications in *Citrus* spp.	An et al. 2013 IN: CARDI (2016)
Grape vine	-	Improvement of important traits in grape vine	**Basic research**: Identification of grape vine promotors and characterization of their expression profiles	ESPINOZA et al. (2013)
Maize	Overexpression of Rusbisco Activase (Rac)	Improvement of the photosynthetic capacity of maize to increase yield and adaptation to harsh climatic conditions	**R&D**: Evaluation of an intragenic maize model compared to conventionally bred cultivars	ALMERAYA&SANCHEZ-DE-JIMENEZ (2016)
Potato	Virus resistance (Potato virus Y)	Overexpression of eIF4E mRNA and protein, which confers resistance to PVY, using site-directed mutagenesis	**R&D**: Engineering virus resistance using a modified potato gene (*de novo* allele of the potato orthologue of the *pvr1* gene of pepper)	CAVATORTA et al. (2011)
Potato	Virus resistance (Potato virus Y)	Overexpression of wild potato *eIF4E-1* variant *Eva1* in potatoes silenced for native *eIF4E-1*	**Basic research**: Identification of the novel gene variant *Eva1*, which confers PVY-resistance to tetraploid potato cultivars	DUAN et al. (2012)
Potato	Reduced acrylamide-forming potential	Silencing of asparagine synthetase-1 (StAst1) to reduce acrylamide-forming potential in tubers	**R&D**: Investigation of the yield drag observed in field trials (associated with the silencing of StAst2, but not StAst1)	CHAWLA et al. (2012)

7.9 Annex 9: Applications of Transgrafting

Table 15: Transgrafting applications retrieved from scientific literature (2015-2017)

*Transgrafting categories indicated in column 1: (1) Transgrafting (incl. heterografting, i.e. grafting involving parts from different plant species), (2) non-GM grafting with traits of interest for further GM developments, (3) Mutation grafting, however involving traits also used in transgrafting experiments, (4) Micro-grafting for regeneration and graft-propagation, (5) Grafting of GM scion on special non-GM rootstock for early fruiting (as methodology to test GM effect in fruit) or to facilitate field testing, (6) Graft-inoculation to transfer pathogen.

Trans-grafting category *	**Crop Plant Rootstock**	**Crop Plant Scion**	**Trait/Characteristic of GM Plant (used for grafting)**	**Conferred Trait/spec. Characteristic of transgrafted Plant**	**Modification, Target Gene(s), Gene Constructs, Approach**	**Purpose of the Study**	**Reference**
1	Tobacco (GM & WT) Apple (GM)	Tobacco (WT & GM) Apple (WT)	Tobacco: early flowering; Apple: less sensitive to short-day-induced dormancy; significantly increased maximum photochemical efficiency	Tobacco scion: shortened time to flowering Apple: delayed senescence and leaves have significantly higher Fv/Fm and chlorophyll content values.	Using transgene from pear (*Pyrus communis*) 355:PcFT2 & yellow fluorescent protein (YFP) reporter gene 355:PcFT2-YFP with 35S promoter to induce overexpression. Tobacco: reciprocal grafting and WT/WT control. Apple: WT/GM	**Basic research**: Into synchronisation of reproductive development. To test novel pear FLOWERING LOCUS T (FT)-like genes, here PcFT2, and their effects on flowering in annual and perennial plants, using tobacco and apple as model plants.	FREIMAN et al. (2015)
1	Apple (M.26: non-GM and M.26/T166: GM)	Apple cultivar 'Royal Gala'	Cold/freeze tolerance, short photoperiod response (early dormancy, late flowering)	Reduced growth and delayed flowering (but no cold hardiness or dormancy effect). Not all traits/characteristics transferred	Overexpression of a peach PpCBF1 gene in apple (CBF = C-repeat binding factor)	**Basic research/R&D**: To determine physiological and morphological effects transmitted to scions that were grafted on a transgenic rootstock overexpressing CBF1 in the field for 3 years.	ARTLIP et al. (2016)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Bottle gourd (*Lageneraria siceraria* Standl.). - GM	Watermelon (*Citrullus vulgaris* Schrad.)	Cold tolerance (no other horticultural traits found in GM vs non-GM rootstock)	Cold tolerance,	GM rootstock expressing Arabidopsis CBF3/DREB1A gene (C-repeat binding factor (CBF). CBF acts as transcription factor in cold-response pathway)	**R&D**: Development of cold resistance rootstock for melon production, to test for transfer of CBF3 gene to scion	CHO et al. (2017)
1	Bottle gourd (*Lageneraria siceraria*) - GM & WT	Watermelon (*Citrullus vulgaris*)	Salt tolerance	Salt tolerance	Bottle gourd expressing the Arabidopsis H+-pyrophosphatase AVP1 gene inducing salt-tolerance. Non-GM watermelon scion grafted onto bottle gourd rootstock (homozygous lines: GM T-2 as well as WT)	**R&D**: To improve agronomic traits of watermelon and for this to develop and test the use of transgenic bottle gourd as salt-tolerant rootstock	HAN et al. (2015a)
1	Cherry	Sweet Cherry	Virus resistance	Virus resistance	-	**R&D:** Development of virus resistant cultivar	SONG&WALWORT (2015)
1	Cherry Colt (GM: riT-DNA Colt)	Sweet Cherry cv. Lapins. & Colt wt	Dwarfing, reduced canopy size	Dwarfing, reduced canopy size; also longer leaf retention in autumn	Using cherry Colt (modified with riT-DNA of *Agrobacterium rhizogenes*) as a rootstock resulted in a number of plants with reduced canopy	**R&D**: To further reduce the canopy size of sweet cherries (10-year field trial) via a suitable rootstock	RUGINI et al. (2015)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Grapevine (*Vitis vinifera*) – GM	Grapevine (Vitis vinifera) – WT	Immunity to Pierce's disease (PD) caused by *Xylella fastidiosa* in grapevine: immunity maintained for over 10 years in greenhouse, but. breakdown after 5 years under field conditions	Immunity to PD under greenhouse conditions, breakdown after 5 years under field conditions	Overexpression of transgenic antimicrobial lytic peptide gene LIMA-A. Provided immunity in greenhouse (10y), but not in the field. In field breakdown of immunity within 7 years for GM plant and 5 years for grafted plant (GM rootstock)	**R&D**: To develop disease resistant rootstock that will transmit resistance to non-GM grafts in the field.	LI et al. (2015)
1	Potato (*Solanum tuberosum*) (1) GM: with 35S:GFP (2) WT	Tobacco (*Nicotiana benthamiana*) GM	GM Tobacco: donor of RNAs for transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS)	Potato: TGS: reduced expression of GFP reporter or CBSSI in sprouting micro tubers and next generation tubers, the latter resulting in lower amylose (40%)/higher amylopectin levels in starch	GM potato with 35S:GFP construct, producing GFP (green fluorescent protein). Grafting with GM tobacco scions producing (in phloem companion-cells) a) siRNA (TGS) signals (Co35SpIR & CoGBpIR) for methylation of the GFP transgene and the endogenous Granule-bound starch synthase I (GBSSI) gene; and b) RNA for PTGS (CoGFPIR construct): PTGS reduced GFP expression in first generation only	**Methodology/R&D**: To obtain/induce heritable gene silencing through RdDM by hetero-grafting with GM scion as siRNA donor. Testing method with GFP (green fluorescent protein) and applying the approach for a gene of agronomical interest, here GBSSI, for reduced amylose starch content. Model for vegetative/clonal propagated crops	KASAI et al. (2016b)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Sweet potato (*Ipomoea batatas* cv. Tainting 57) (GM & WT)	Sweet potato (GM & WT)	GM scion: donor of signal for inducing IPO (Ipomoelin) expression in WT rootstock after wounding of scion	Enhanced wounding response (production of IPO and lignin) in OE/WT plants and WT/OE. Reduced wounding response in RNAi/WT	Analysis of IbpreproHypSys, a small signaling peptide, the precursor of IbHypSys, linked to wound-inducible IPO expression by two approaches: 1) overexpressing IbpreproHypSys 2) silencing IbpreproHypSys via RNAi	**Basic research**: Investigating the wounding response signalling pathways: in particular the signal transduction and regulation of IbpreproHypSys, resulting in activation of lignin biosynthesis and protection from insects	LI et al. (2016d)
1	Tobacco (GM & WT)	Tobacco (WT & GM)	(1) GFP (2) GFP fused to protein of interest, here KN1	(2) Detectable long-distance transport. (3) When nGM plant WT/PbKN1-GFP is also NtMPB2C-silenced: enhanced long distance transport	Using pear KN1 gene fused with GFP marker in tobacco (35S:GFP-PbKN1) as well as agroinfiltration of WT/PbKN1-GFP plants with an RNAi silencing construct for the movement protein binding protein (MPB2C)(known to bind KN1 and some viruses)	**Basic research**: On movement, role and regulation of the homeodomain transcription factor 'KNOTTED1 (KN1)-like homologous box' (KNOX) in pear and tobacco and its interaction with and possible regulation by MPBC2 protein	DUAN et al. (2015)
1	Tobacco (GM)	Tomato & tobacco: susceptible to TMV & ToMV respectively	Tobacco Sd1 rootstocks: Virus resistance and siRNA donor	Resistance to tobamoviruses (here ToMV & TMV)	GM rootstock: silencing of NtTOM1 and NtTOM3 genes required for tobamovirus multiplication with hairpin RNAi. Same signal will silence tomato homologs (LeTH3 and LeTH1)	**R&D**: To induce virus resistance in non-GM scions via RNAi, to obtain products without GM modifications	ALI et al. (2016)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Tobacco (*N. tabacum*) – GM	Tobacco (*N. tabacum*) – WT	Heat and cold tolerance	Heat and cold tolerance	Overexpression of papaya transcription factor genes (CpRap2.4a, CpRAap2.4b, CpRap2.1 and CpRap2.10) involved in triggering abiotic stress tolerance. To visualise movement and location, Rap2 proteins were fused with the GFP reporter protein	**Basic research**: To understand the long-distance signaling in response to abiotic stress and the role of the AP2/ERF gene superfamily (transcriptional regulators which serve a variety of functions in plant development and responses to biotic and abiotic stimuli)	FIGUEROA-YANEZ et al. (2016)
1	Tobacco (*N. tabacum*) – GM & WT	Tobacco – WT & GM	Cytokinin (CK) overproduction to prime plant for enhanced response to pathogens (here Potato virus Y^{NTN} (PVY^{NTN}))	GM grafts: tolerance to PVY^{NTN}	Gene construct (P*ssu-ipt*) for CK overexpression. Grafting: stimulates production of compounds such as phenolic acids, thus synergistic effect of both grafting and high CK led to PVY^{NTN} tolerance in transgenic grafts	**Basic research**: To find out if and how the susceptibility of tobacco to PVY^{NTN} is affected by priming caused by two different factors, such as overproduction of endogenous CK and grafting	SPOUSTOVA et al. (2015)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Tobacco (*Nicotiana benthamiana & N. tabacum*) - GM & WT	Tobacco (*Nicotiana benthamiana* & N. *tabacum*) - GM & WT	Different GM lines e.g. expressing and donor of: P20 protein; satBaMV RNA/protein; RNAi to reduce fibrillarin expression	Grafted plants as test system for studying satRNA behaviour	Numerous constructs and transgenic lines including: (1) $SUC2_{pro}$:P20 expressing the P20 protein (of the Bamboo mosaic virus) under the phloem companion cell-specific SUC2 promoter (2) $SUC2_{pro}$:P20:eGFP (for visualisation) (3) $SUC2_{pro}$:P20:eGFP (4) $35S_{pro}$:P20:eGFP (expression in all cell types) (5) Fibrillarin RNAi construct to reduce endogenous fibrillarin expression	**Basic research**: To understand how satRNAs (parasites of viruses, depending on helper viruses for replication, encapsidation and spread) are interacting with viruses and cellular machinery and how they undergo intracellular or intercellular trafficking. To determine whether satBaMV (Bamboo mosaic virus (BaMV)-associated satRNA) can move systemically in the absence of a helper virus	CHANG et al. (2016)
1	Tobacco (*Nicotiana tabacum*) GM & WT and *Arabidopsis thaliana* GM & WT	Tobacco (*Nicotiana tabacum*) GM & WT Arabidopsis GM & WT Grafting in all com-binations, mostly with-in species and GM rootstock with WT scion	Using production of misshaped pollen in anthers and decreased fertility as a reporter system for mRNA mobility	Various phenotypes	Transformation with constructs that produce reporter transcripts harboring tRNA-like structures (TLSs). Such TLS are enriched in the phloem stream and in mRNAs transmitted over graft junctions. - using Arabidopsis variant with DISRUPTION OF MEIOTIC CONTROL1 ($_{DN}$DMC1) as well as gene constructs of $_{DN}$DMC1 in combination with various motives - as well as full $tRNA^{Met}$ in tobacco and Arabidopsis	**Basic research**: To investigate the RNA motives triggering and enabling the mobility and the extent of long distance transport as well as the potential of transported mRNAs to be translated into functional proteins after transport	ZHANG et al. (2016c)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Tomato – GM	Tomato – WT	Cucumber mosaic virus (CMV)resistant rootstock as donor of ihpRNAs	Virus resistance to WT-scions (CMV)	Five RNAi constructs expressing intron-spliced hairpin RNAs (ihpRNA) were used to silence five CMV genes). Virus resistance was achieved and transferred to scions	**Methodology R&D**: To improve method for rootstock propagation and to provide virus resistant transgenic rootstock for non-GM production of grafted fruit	BAI et al. (2016)
1	Tomato – GM	Tomato – WT	High temperature tolerance. Donor of RNA silencing	High temperature tolerance	Two constructs with inverted repeat RNAs from fatty acid desaturase gene (LeFAD7) expressed in transgenic rootstock used for gene silencing in non-GM scion. FAD7 affects the composition of membrane lipids and thus temperature sensitivity	**R&D**: To investigate graft transmission of RNA silencing for high temperature tolerance	NAKAMURA et al. (2016)
1	Tomato – GM	Tomato (WT) (highly virus susceptible lines)	Virus resistance and donor of siRNA.	No transfer of immunity to tomato yellow leaf curl virus (TYLCV)	PTGS approach using hairpin constructs derived from three different conserved viral sequences as a single insert	**R&D**: To provide protection of non-transgenic scions against TYLCV	LEIBMAN et al. (2015)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Tomato (*Solanum lyco-persicon)* GM & WT	Tomato (*Solanum lycopersicon*) WT & GM	Reduced stem xylem area, stem & root water conductance, whole plant transpir ation, wilting phenotype	Reciprocal and double grafting: Wilting phenotype detected only in RNAi/WT grafting.	Transformation of tomato with reverse fragment of SlFRK3 (fructokinase) gene for RNAi Grafting: reciprocal grafting (WT/RNAi & RNAi/WT) and double grafting (WT/RNAi/WT) or (RNAi/ WT/RNAi) with three different segments: rootstock, short stem graft, upper scion to analyse for imposing growth effects & wilting	**Basic research**: To study the role of *Sl*FRK3, the only plastidic fructokinase, as well as its interaction with the cytosolic FRK2 in xylem development and water conductance	STEIN et al. (2016)
1	Tomato (*Solanum lycopersicum*) – GM	Tomato (*Solanum lycopersicum*) – WT	Donor of RNAi signal; expressing a wide range of altered phenotypes, e.g. altered growth rate (dwarfism) and altered flowering (incl. male sterility)	Increased vigour of WT/RNAi and its WT progeny)	Use of MSH1-RNA interference construct to silence MutS HOMOLOG1 (MSH1) in GM rootstock and non-GM scion and to induce a heritable epigenetic effect	**Basic research**: Investigating the role of MSH1 to affect developmental reprogramming of the plant and the feasibility of epigenetic engineering and breeding in a crop plant.	YANG et al. (2015)
1	Tomato (WT strain carrying the I-2 resistance gene)	Tomato GM also: pathogen *Fusarium oxysporum* with Avr2 knock-out	Producing Avr2 effector protein a) cytosolic or b) secreting it	No immune response triggered by Avr2 presence in sap	Transgenic tomatoes expressing either a secreted (Avr2) or cytosolic Avr2 (Delta spAvr2) protein, also utilising an Avr2 knock-out *Fusarium* (Fol Delta Avr2)	**Basic research**: Into pathogen-host mechanisms, and in particular the virulence factor Avr2 from *Fusarium oxysporum.* Invatigating whether the uptake of effectors is a host autonomous process, and where immune response is triggered	DI et al. (2016)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
1	Walnut (*Juglans regia*) GM	Walnut (non-GM)	DsRED marker		Utilising DsRED marker gene to test for gene translocation and presence of mRNA across the graft junction	**Methodology/Basic research**: To establish an *in vitro* grafting methodology to examine gene translocation and mRNA translocation from GM rootstock to WT scion	LIU et al. (2017)
2	Rice (*Oryza sativa* L.) - BRRI 33 (Arsenic tolerant strain – non-GM)	Rice (*Oryza sativa* L.) - BRRI 51 (Arsenic-sensitive strain)	Arsenic tolerance (non-GM)	Arsenic (As) tolerance & As-free rice production	Utilising non-GM Arsenic tolerance strain for grafting experiments.	**Basic research/R&D**: To understand the molecular mechanisms of As tolerance (the origin of As tolerance signal by reciprocal grafting of contrasting genotypes) and to develop an As-free transgenic rice	BEGUM et al. (2016)
3	*Arabidopsis* – msh1 mutation & WT	*Arabidopsis* – WT & msh1 mutation	Altered plant growth behaviour and CG methylation	F1 from WT-Col-0/msh1: enhanced growth phenotype; inheritable altered CG methylation (not in msh1/WT)	Utilising a mutation strain (msh1) with downregulation of MSH1 (MutS HOMOLOG1) for grafting	**Basic research**: Showing that MSH1 is directly involved in heritary epigenetic changes in the genome and affecting the methylome	VIRDI et al. (2015)
4	Avocado (*Persea americana* Mill.)	n/a	n/a	n/a	Expression of the marker genes gfp, DsRed and a *gfp-gus* fusion gene, respectively	**Methodology**: Recovery of plants & marker gene system	PALOMO-RIOS et al. (2017)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
4	Citrange cv Carrizo (*Citrus sinensis* Osb. x *Poncirus trifoliata* L. Raf.)	n/a	n/a	Marker/reporter genes (nptII & GFP)	n/a	**Methodology**: Methods for transformation and regeneration	WU et al. (2016a)
4	Citrus	Citrus	n/a	Not relevant	n/a	**Methodology**: Transformation with tissue culture explants for easier R&D	ORBOVIC et al. (2015)
4	Grapefruit (*C. paradisi*) & sweet orange (*C. sinensis*)	Grapefruit (*C. paradisi*) & sweet orange (*C. sinensis*)	n/a	Reporter genes (*gus* & *gfp*)	Reporter genes for identification of transgenic shoots	**Methodology**: To establish transformation methodology that would allow developing disease resistance (canker and greening bacterial disease)	WU et al. (2015)
4	Groundnut (*Arachis hypogaea* L.)	Groundnut (*Arachis hypogaea* L.) - transgenic	n/a	Not relevant	n/a	**Methodology**: Methods for *Agrobacterium*-mediated transformation and regeneration	TIWARI et al. (2015)
4	Kumquat (*Fortunella crassifolia* Swingle.)				Grafting used to support regenerating transformants.	**Methodology**: To improve transformation efficiency of kumquat	YANG et al. (2016)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
4	Pommelo (*Citrus maxima*)			Marker gene: herbicide tolerance (hygromycin)		**Methodology**: For efficient in planta transformation (*A. tumefaciens*)	Zhang, Zhang et al. 2017
5	Apple (dwarf clonal rootstock)	Apple GM	Delayed spoilage of fruit	GM fruit: (prolonged shelf life) Transgrafted plant: early fruiting to allow quicker evaluation	1) Silencing one of the key genes of the ethylene biosynthesis pathway, apple 1-aminocyclopropane-1-carboxylate (ACC) oxidase, in order to prolong shelf life of fruit 2) Silenced scion rafted on dwarf clonal rootstock N62-396 for earlier fruiting	**R&D**: Towards prolonged shelf life in apples (35S:uidA only known to work in tomatoes)	KLEMENTJEVA et al. (2016)
5	Grapevine (*Vitis vinifera*) - 'Harmony' line	Grapevine (*Vitis vinifera*) 'Thompson Seedless' line GM	Fungal resistance	Fungal resistance (to gray mold and powdery mildew)	Two endochitinase (ech42 and ech33) genes and one N-acetyl-beta-d-hexosaminidase (nag70) gene from biocontrol agents related to *Trichoderma* spp. were used (grafting used for practical purposes)	**R&D**: To develop disease resistant grapevine, immune to the fungi *Botrytis cinerea* (gray mold) and *Erysiphe necator* (powdery mildew) and to further select and characterise the best transgenic lines	RUBIO et al. (2015)
6	Cassava	n/a	n/a	n/a	To test effectiveness of RNAi transgene method & events. Using an inverted repeat construct (p5001) derived from coat-protein (CP) sequences of CBSV and UCBSV fused in tandem	**R&D**: To achieve resistance to cassava brown streak disease (CBSD) caused by cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) (transmitted by whitefly)	BEYENE et al. (2017)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
6	Citrus - Carrizo	Citrus Carrizo	Thionin-induced citrus canker and Huanglongbing resistance	Reduced disease symptoms of citrus canker and Huanglongbing (HLB or citrus greening disease) and reduced bacterial growth.	Overexpression of a modified plant thionin.	**R&D:** Towards developing resistance in citrus to both HLB and citrus canker caused by *Candidatus Liberibacter asiaticus*, through overexpression of a modified plant thionin	HAO et al. (2016)
6	Lime	Lime	Resistance to citrus tristeza virus (CTV)	Resistance to CTV	RNAi against three viral genes encoding silencing suppressors. Reported that resistance was achieved	**R&D**: To show it is possible to achieve full resistance to CTV in a highly sensitive citrus host by targeting simultaneously the three viral silencing suppressors through RNAi	NURIA et al. (2015)
6	Peach (PPV-D infected)	Plum (GM & non-GM)	Sharka resistance	Sharka resistance (plum pox virus – PPV).	Hairpin h-UTR/P1 construct	**R&D/Methodology**: to produce several transgenic plum lines resistant to PPV, to set up an efficient and reliable in vitro PPV resistance test and to compare it with the standard greenhouse evaluation	GARCÍA-ALMODÓVAR et al. (2015)
6	Plum (*Prunus domestica* L.)	n/a	n/a	PPV resistance	Plum pox virus resistance via transformation with intron-hairpin-RNA construct	**R&D**: To test five independent transgenic plants for resistance through five dormancy cycles	SIDOROVA et al. (2016)

Trans-grafting category *	Crop Plant Rootstock	Crop Plant Scion	Trait/Characteristic of GM Plant (used for grafting)	Conferred Trait/spec. Characteristic of transgrafted Plant	Modification, Target Gene(s), Gene Constructs, Approach	Purpose of the Study	Reference
6	Plum (*Prunus domestica* L.)	Plum GM	Sharka (PPV) resistance	PPV resistance	Testing of PPV resistance (based on RNAi) through graft-inoculation.	**R&D**: Protection against sharka disease (plum pox virus) and evaluation of transgenic plum *Prunus domestica* L., clone C5 (cv. HoneySweet (12 year filed trial)	POLÁK et al. (2017)
6	Plum (*Prunus mariana*) – infected	Plum (*Prunus domestica*) GM	Sharka (PPV) resistance	Sharka resistance – Plum Pox Virus (PPV)	Transformation with combined PPV-P1+PPV-coat protein (CP) construct leading to accumulation of PPV-specific siRNAs	**R&D/Basic research**: To create and test resistance to Sharka and to test constructs	RAVELONANDRO et al. (2015)

7.10 Annex 10: Applications of Agroinfiltration

Table 16: Agroinfiltration applications retrieved from scientific literature (2011-2017)

Plant	**Purpose of the Application, Approach, Target Gene(s)**	**Purpose of the Study**	**Reference**
Nicotiana spp.	Agroinfiltration Triggering of HR	**Basic research**: Evaluation of the potential of three different tomato bushy stunt virus (TBSV) proteins (P33, P92, and P41) to elicit HR	ANGEL&SCHOELZ (2013)
Black gram	Agroinoculation Infection with cloned DNA-A (clone MF2, JQ398669) and DNA-B (clone MF1, JQ398670) components of MYMV-ND	**Basic research**: Evaluation of the responses of black gram germplasm to mungbean yellow mosaic virus (YMD) Identification of potential sources of resistance	BAG et al. (2014)
Grapevine	Agroinfiltration Transient silencing of the grapevine gene VvPGIP1	**Basic research**: Study gene function Testing the possibility to silence grapevine genes by agroinfiltration of RNAi constructs	BERTAZZON et al. (2012)
Water melon, zucchini	Agroinfection Co-infection using clones of WmCSV and ToLCPMV	**Basic research**: Analysis of watermelon chlorotic stunt virus (WmCSV) and tomato leaf curl palampur virus (TolCPMV)	ESMAEILI et al. (2015)
Nicotiana benthamiana	Agroinoculation With TVBMV	**Basic research**: Analysis of gene expression patterns in wild-type tobacco vein banding mosaic virus (TVBMV)-infected (T-WT) and attenuated TVBMV mutant infected (T-HCm) *N. benthamiana*	GENG et al. (2016)
Nicotiana benthamiana	Agroinfiltration Transient expression of WRI1	**Basic research**: Analysis of the transcriptional transitions in leaf tissue upon induction of oil synthesis by WRI1.	GRIMBERG et al. (2015)
Nicotiana benthamiana	Agroinfiltration Cry1Ac, *Brassica oleracea* proteinase inhibitor (BoPI),	**Basic research**: Determination of the predictive effects of overexpressing two different candidate insect resistance genes in plants	LECKIE&STEWART JR (2011)
Nicotiana benthamiana Tomato	Agroinfiltration, agroinjection Transient expression of the GUS reporter gene driven by the TPSI1 promoter	**Basic research**: Phosphate starvation-induced gene (TPSI1) from tomato (*Solanum lycopersicum* L.) was used to reflect phosphate status in plants	LIN et al. (2016)

Plant	Purpose of the Application, Approach, Target Gene(s)	Purpose of the Study	Reference
Nicotiana benthamiana	Agroinfiltration RNA1 and RNA2	**Basic research**: Construction of full-length cDNA clones of the tomato chlorosis virus (ToCV) genome (RNA1 and RNA2)	ORÍLIO et al. (2014)
Tomato	Agroinoculation Combining Ty-2 and Ty-3 genes in tomato hybrids	**Basic research/ R&D**: Development of tomato lines, carrying Ty-2 and Ty-3 resistance genes as sources for achieving higher levels of resistance to tomato leaf curl disease	PRASANNA et al. (2015)
Nicotiana benthamiana	Agroinfiltration Transient expression to demonstrate specific and strong recognition of AvrPm2 by Pm2	**Basic research**: Map-based cloning and genome-wide association studies to isolate a candidate for the mildew avirulence gene AvrPm2	PRAZ et al. (2017)
Tobacco	Agroinfiltration	**Basic research**: Study of the expression of *OsXPB2* promoter	RAIKWAR et al. (2015)
Cucurbita spp.	Agroinoculation	**Basic research**: Identification of sources of resistance to tomato leaf curl New Delhi virus (ToLCNDV)	SÁEZ et al. (2016)
Nicotiana tabaccum	Agroinfiltration with cells expressing βC1 protein	**Basic research**: Examination of the suppressor function of ORF βC1 of three betasatellites of plant diseases	SHUKLA et al. (2013)
Various plant species	Agroinoculation	**Basic research**: The partial dimeric construct (*p*Bin-1.4-BCTIRV) was tested to obtain an experimental host range of beet curly top Iran virus (BCTIRV)	SOLEIMANI et al. (2013)
Tomato wild relatives	Agroinoculation with TYLCV-IL	**Basic research**: Germplasm screening Identification of two tomato yellow leaf curl disease (TYLCD) resistance sources, EELM-388 and EELM-889	TOMÁS et al. (2011)
Tomato	Agroinoculation	**Methodology/Basic research**: Developing a simple agroinoculation method with an infectious clone of tomato yellow leaf curl virus (TYLCV) and evaluation of differences in TYLCV resistance among tomato cultivars	YAMAGUCHI et al. (2013)
Solanaceous species	Agroinoculation	**Methodology**: Development of method for virus-induced gene silencing	ZHANG et al. (2013)

7.11 Annex 11: Applications of CENH3-mediated Haploid-Induction (HI)

Table 17: Research addressing Haploid induction in scientific literature (2011-2017)

Plant	**Purpose of the application, Approach, Target Gene(s)**	**Purpose of the Study**	**Reference**
Arabidopsis thaliana	Induction of point mutations in CENH3 genes by TILLING	**Basic research**: Research to identify CEN H3 mutations with impaired centromeric loading and the ability to induce haploid induction *in vivo* by chromosome elimination during early zygotic embryogenesis in crosses of CEN H3-mutant females x wildtype male plants	(KARIMI-ASHTIYANI et al. 2015)
Arabidopsis thaliana	Use of haploid induction by GFP-CEN H3 tailswap for QTL analysis	**R&D**: Application of the GFP-CEN H3 tailswap based approach for haploid induction for fast-track establishment of DH lines for QTL analysis	(SEYMOUR et al. 2012)
Arabidopsis thaliana	Development of an *in vivo* method of haploid induction using GFP-CEN H3 tailswap transgenic constructs	**Methodology**: Development of protocols for *in vivo* haploid seed production employing an improved GFP-tailswap strain called Seed GFP haploid inducer (avoiding tissue culture-based methods)	(RAVI&BONDADA 2016)
Arabidopsis thaliana	Functional replacement of a Cen H3-null mutation in *Arabidopsis thaliana*, using CEN H3s from increasingly distant relatives	**Basic research**: Cen H3 variants from other species (incl. maize) can complement a Cen H3-null mutation in Arabidopsis, however induce missegregation (aneuploidy, haploid induction) in crosses with WT Arabidopsis	(MAHESHWARI et al. 2015)
Arabidopsis thaliana, Maize, Rice	Identification of genes involved in plant meiosis	**Basic research**: Review of functional characterization of meiosis specific genes for potential use in plant breeding	(CRISMANI et al. 2013)
Maize	Development of methods for production of doubled haploid plants using available haploid inducer lines	**Methodology/R&D**: Development of locally adapted haploid inducer lines and methods which are improved in terms of survival, maturity and fertility compared to treatment with colchizine for the production of doubled haploid	(ASLAM et al. 2017)
Maize	*In vivo* haploid induction in tropical maize lines	**Methodology/R&D**: Adaptation of *in vivo* methods for haploid induction for tropical maize lines based on existing inducer lines (KEMS) and a colour based selection system for hapoids (R1 Navajo)	(COUTO et al. 2015)
Maize	*In vivo* haploid induction in subtropical maize lines	Methodology/R&D: Adaptation of available haploid inducer lines for the generation of doubled haploid varieties in subtropical maize varieties	(DANG et al. 2012)
Maize	Establishment of CEN H3 tailswap lines for *in vivo* maternal chromosome elimination	**R&D**: Research to translate results from pilot studies in Arabidopsis (RAVI&CHAN 2010) to maize to establish a system for *in vivo* haploid induction with female inducer lines	(KELLIHER et al. 2016)

Maize	Identification of mutations in MATRILINEAL (MTL), a pollen-specific phospholipase, to trigger *in vivo* haploid induction	**Basic research/R&D**: Research to elucidate the mode of action of genes involved in haploid induction in male lines used for *in vivo* doubled haploid production	(KELLIHER et al. 2017)
Maize	Selection system for haploid seeds produced from *in vivo* haploid induction	**Basic research/Methodology**: proof of concept study for the development of an universally applicable system based on increase oil-content for the selection of haploid seeds produced from high-oil inducing male lines	(MELCHINGER et al. 2013)
Maize	Development of haploid inducer lines adapted to genetic backgrounds of tropical varieties	**R&D**: Establishment of adapted haploid inducer lines for use in breeding programs of tropical maize lines based on doubled haploid technology	(PRIGGE et al. 2012)
Barley	Production of doubled haploids subsequent to GE-applications	**Methodology**: Development of methods for GE by TALEN in embryogenic pollen cultures for production of genetically modified doubled haploid plants	(GURUSHIDZE et al. 2017)
Onion	Transcriptomic characterization of a homozygous double haploid line CUDH2107	**Basic research/R&D**: Functional characterization of vegetative and reproductive tissues of a DH onion line for use as reference for further research	(KHOSA et al. 2016)
Wheat	Methods for haploid induction in wheat by wide-crosses with maize lines	**Methodology**: Review of procedures for DH production of durum and common wheat via wide hybridization with maize and optimization of protocol for haploid induction in wheat	(NIU et al. 2014)
Brown mustard (*Brassica juncea*)	Development of haploid induction lines in brown mustard by RNAi knock-down of endogenous CEN H3 alleles and complementation with transgenic GFP-CENH3-tailswap constructs	**R&D**: First report demonstrating that the principles for CENH3 engineering established in Arabidopsis (RAVI&CHAN 2010) could be used to develop HI lines in a polyploid crop	(WATTS et al. 2017)

7.12 Annex 12: Results of the Expert-Workshop “Risk assessment of plants generated with New Techniques“, May 3rd 2017, BfN, Bonn

7.12.1 Introduction

This workshop was conducted in the framework of a research & development project commissioned by the German Federal Agency for Nature Conservation (BfN), which is carried out by Environment Agency Austria (EAA). The aim of the workshop was to discuss current and future developments in the field of application of New Techniques (nGM) in plant development as well as risk assessment issues associated with nGM applications.

As an introduction the BfN presented the background of the project, highlighting the current discussions on the subject taking place in Germany, e.g. in the framework of a national dialogue hosted by the German Ministry of Agriculture, which is addressing a broad range of aspects concerning the application of nGMs, particularly of Genome Editing approaches. In addition the recent inquiries by companies and universities concerning the regulatory requirements for field release of plants developed with nGM led to a discussion concerning particular nGM applications.

Presentation “Risk assessment of plants generated with New Techniques, part 1”

EAA presented the workshop aims, in particular the objective to discuss recent technological developments concerning the application of specific nGMs, the respective state of knowledge concerning such applications and the identification of key aspects of nGM applications with relevance for biosafety. The presentation included the list of New Techniques which are addressed in the current project:

- Genome editing approaches: CRISPR, TALENs, ZFNs, meganuclease, targeted chemical modification of specific DNA bases, ODM, MAGE
- Approaches to modify gene expression: RNA dependent DNA methylation, CRISPR-directed modification of methylation, Virus-aided gene expression, RNAi-based gene silencing, CRISPR-directed transcriptional regulators
- Cisgenesis/Intragenesis
- Transgrafting
- Agroinfiltraion: agroinfiltration, agroinfection, floral dip
- Reverse breeding
- Haploid induction: CenH3-based approaches

Presentation “Risk assessment of plants generated with New Techniques, part 2”

In a second presentation EAA presented technical issues for discussion. Those were risk assessment questions regarding characteristics of the used technology (e.g. issues due to the molecular modifications due to nGMs) and issues resulting from the developed trait(s).

On the other hand unintended effects associated with the nGM and respective risk assessment issues need to be addressed.

7.12.2 **Discussion**

Regulatory issues

The first part of the discussion addressed general issues concerning the regulation of nGMs. It was discussed whether different approaches to regulation may be based on similarities and differences between nGMs and conventional breeding methods. In this respect participants noted that recent developments may weaken this distinction: Whereas some nGMs like Genome Editing approaches were developed with the aim to introduce targeted mutations, also recent conventional mutation approaches are aimed to generate and select mutations in specific genomic loci.

Another discussion point was the interpretation of the existing GMO legislation in the EU, which is covering an open list of techniques with some specific exceptions (e.g. conventional breeding, mutagenesis). However, it was also pointed out during the discussion that the novelty of GM technology was not the only reason for the development of specific legislation. It was noted that another reason for introducing specific legislation for GMOs was the introduction of novel genetic elements, e.g. of recombined sequences of foreign origin. Some applications of nGMs however will not lead to the introduction of recombined foreign genetic material, but rather to introduce random or directed genetic changes at specific genomic locations. It was noted that such changes can lead to the generation of new phenotypic traits with a different potential for adverse effects. However, such changes may in principle also arise by natural processes (transposon mediated changes or transformation by naturally occurring agrobacteria) or conventional mutation techniques.

The further discussion also addressed the issue whether new varieties developed by conventional breeding approaches should be treated differently as plants produced by nGMs. The discussion touched on the issue, whether natural processes and/or biotechnological interventions (through application of nGMs) may result in risks that need to be assessed. It was noted that all genomic changes (intended as well as unintended changes) need to be considered in this respect. Participants also noted that experience with the cultivation of plants with respective characteristic and observations made during the breeding process need to be considered whether a history of safe use can be inferred.

Important issues identified were:

- The potential of new methodological developments as regards nGMs, enabling specific applications (e.g. by development of particular traits or by multiple changes in complex metabolic pathways).
- The specificity of nGM approaches to introduce particular genetic changes and the relevance of specificity as regards the estimation of potential adverse effects.
- The specific nature of the introduced change(s) and the level of experience (familiarity) with such changes.

7.12.3 **Relevant developments, current & future applications**

It was noted that the toolbox of nGMs is evolving and that nGMs can be used in combination (e.g. RNA depended methylation with grafting, CRISPR/Cas and ODM as donor/template sequence). It was also noted that the development of more complex traits will likely follow.

nGMs are implemented to speed up the development process, e.g. to reduce the number of necessary breeding steps and/or shorten the time needed for a breeding step (e.g. by induction of early flowering in accelerated breeding applications).

Application of CRISPR-based techniques can also speed up breeding processes considerably. CRISPR-technology is user-friendly and – different to other nGMs – its application is not limited to a few applicants, who possess specialised knowledge and experience. In addition CRISPR technology is easily adapted to different genomic target sequences and facilitates the parallel or serial modification of several different genomic target sites. It was noted that with CRISPR-based techniques the introduction of specific modifications is less of a challenge than the associated process of regeneration of modified plants.

Specific considerations were noted for the following nGM applications:

CRISPR-based techniques:
CRISPR technology is rapidly evolving (e.g. developed to be more precise with reduced off-target activity, further developed to facilitate different applications, particularly for different types of genomic modifications incl. base editing, for modification of gene expression, etc.). However, it was noted that other nuclease-based systems, like ZFN, play an important role in their own right. Although ZFN-based GE is more difficult to customise and more expensive, it is a very robust and proven technique.

DNA-free GE:
Such applications do not include a step where a SDN-expressing DNA construct is integrated into the target cell. Therefore DNA-free GE applications are developed also for application in plant breeding.
However, certain limitations need to be considered for use in plant cells: DNA-free GE reagents can only be introduced into plant protoplasts. Thus a technical bottleneck is the availability of methods to produce, modify and regenerate protoplasts into modified plants. Protoplast transformation methodology is not established for all species and can lead to generation of higher numbers of unintended mutations. DNA-free GE methodology therefore associated with a newly sparked interest in the further development of protoplast technology in plant breeding.

Allelic replacement by CRISPR:
SDN-3 applications are important for breeding, however the selection of successful integration events is difficult because of the low frequency of HR-directed integration compared with NHEJ repair events.

Reverse breeding RB):
RB is included in most of the previous studies as an example for an nGM which generates GM intermediates, but aims at the production of plants, which finally should contain no GM traits any more.
However, it was indicated that the practical relevance is of RB is limited to species with a low chromosome number and thus only few applications of RB have been developed so far: Apart from proof of concept studies in Arabidopsis the system was only developed for cabbage species. It was noted that accelerated breeding (AB) is an nGM application which may be of higher relevance for the near future than RB (AB is considered to be applicable

with a larger number of plant species and is particularly relevant for biennial plant species with a longer generation time).

Haploid induction (HI):
HI is considered of practical relevance for major crop plants as well as for plant species, for which less genomic information is available. HI is useful as a tool for the reduction of genomic complexity, which is advantageous for breeding.

RdDM:
It was noted that typically the modification of expression by RdDM is not stably inherited in offspring for more than a few generations. This is not particularly suitable for the development of agricultural applications, where stable products are required.

A focus of nGM applications is the development of plants with modification of multiple genes, e.g. for generating disease resistant plants. It was also noted that with nGMs it is possible to modify (or knock-out) whole gene family at once.

The current approaches to develop potato lines harboring multiple disease resistance genes were presented as an example. Similarly increased protection against pests can be developed by combining different pest resistance factors, since multiple resistance factors are less likely to be overcome by disease or pest adaptation. However, it was noted that the successful development of complex traits requires a lot of knowledge concerning the underlying biology.

It was discussed, that the specific traits developed by nGMs could also influence the acceptance of the technology. Plants with benefits for consumers, e.g. harbouring traits to improve flavour or to reduce allergens, could increase acceptance. The adoption of seedless grapes (developed by conventional approaches) was noted as an example. However, the discussion did not come to a conclusion whether the present examples of apples and mushroom varieties with reduced browning behaviour would be received equally well.

7.12.4 **Knowledge & risk assessment considerations**

The question was raised, whether complete knowledge on the molecular level is needed in order to conduct a risk assessment. Although the aim of science is to acquire more knowledge, complete knowledge is not necessarily needed for risk assessment. It was also pointed out, that a lot of knowledge is acquired during development and breeding. Knowledge is also acquired from field data and experiences with GMOs so far.

It was stated, that sometimes not the characteristics of the modified plant itself are problematic, but how it is used e.g. in intensive agriculture.

It was noted that a lot is unknown regarding RNAi, e.g. immunologic responses to siRNA or issues of specificity of effects on target vs. non-target species. In addition bioinformatics analyses are hampered by the fact that relevant genomic information is not available for all predator species, which might be affected.

It is currently assumed that there is no adverse effect on NTOs, if closely related species are unaffected. However, apart from a study conducted by Monsanto, little empirical evidence is available.

It was discussed whether there are differences in specificity matter for risk assessment considerations. The specificity of GE applications would typically be higher than with undirected mutation methods. It was noted that there is no direct link between the degree of specificity of the intervention and the overall risk associated with a respective application. However, increased specificity would be preferred with a view to off-target effects.

The discussion also addressed the issue of detecting off-target effects. Although gene sequencing is now less expensive, for some species no reference genome exists. For the comparison of sequences respective bioinformatics approaches are needed to detect off-target modifications. It was noted that it is difficult to distinguish off-target effects from natural occurring variation between generations.

7.12.5 **Current activities in various Member States**

Participants presented current activities concerning regulation and biosafety considerations for nGM applications in their Member States. In general it was pointed out, that the expected ruling of the European Court of Justice put activities on the subject on hold in various countries. Future activities will most likely depend very much on the court ruling and its consequences.

However, some fields are actively addressed at present: EFSA commissioned a systemic review on RNAi concerning various topics (e.g. molecular aspects, food/feed safety, different animal groups). The amount of papers found is very extensive. Also in the US a literature review on RNAi was finalised in 2015.

It was noted that at the OECD an information exchange and discussion mechanism for nGM applications was established. Most countries adopted a case-by-case approach and review applications accordingly to define whether they fall under the respective regulatory framework (mostly developed for GMOs).

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