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RNAi-based techniques, accelerated breeding and CRISPR-Cas: basics and application in plant breeding

Executive summary

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1 Introduction

Competent authorities of European Union Member States received questions from stakeholders, due to progress in research and development, on whether certain plant breeding techniques lead to regulated GMOs. Today, this so called “new plant breeding techniques” (NPBT) contain (1) site directed nuclease (SDN) mediated genome editing, (2) oligonucleotide directed mutagenesis (ODM), (3) cisgenesis and intragenesis, (4) RNA-dependent DNA methylation (RdDM), (5) Grafting (on GM rootstock), (6) reverse breeding, (7) accelerated breeding, (8) agro-infiltration, and (9) synthetic genomics (in extension of [1]).

AGES (Austrian Agency for Health and Food Safety) has reported on some of the NPBTs [2, 3]; CRISPR-Cas based genome editing and accelerated breeding are recent developments, thus the coverage in this report. RNAi-based methods are addressed in this report as well, which, particularly because of their potential for engineering pathogen resistance traits, are in the focus of interest again.

This report is intended as an information document for stakeholders in the broader sense. It provides background information on the fundamentals, the state of the technology and the application potentials of these techniques. It describes intended and unintended effects on the plant genome in relation to other plant breeding techniques and biotechnological methods.

The study indicates that the techniques reported on hold great potential for application in plant breeding and cultivar development, and that internationally they are implemented in commercial purposes of breeding.

The legal classification of NPBTs in the EU has large consequences on their application in plant breeding. Thus, it would be of advantage to be decided upon in the near future and in cooperation with the EU partners in order to ensure legal certainty in the EU in respect to application of these techniques in plant breeding and cultivar development.

Information of the public by public authorities in respect to plant breeding and biotechnological methods, their development and application in plant breeding should be an active process and guided by the current state of science technology.

2 CRISPR-Cas

2.1 Introduction

CRISPR-Cas (Clustered regularly interspaced short palindromic repeat – CRISPR associated) based genome editing is a term used to describe the most recent addition to site directed nuclease (SDN) mediated genome editing, in which the nuclease is guided by an RNA component to its target. The number of publications of CRISPR-Cas in the plant sciences reflects its rapid exploration and application in plants (Fig. 2.1 in the study).

2.1.1 Types of targeted genome editing (SDN1,2,3)

Different types of targeted genome editing can be implemented by using SDN technology, including CRISPR-Cas9, to date in particular (Fig. 2.6 in the study): (1a) site specific random mutations, (1b) genomic deletion by placing two DSBs leading to the loss of the genomic region within, (2) site specific pre-defined mutations (by providing a repair template) and (3a, 3b) insertion of larger fragments of ectopic DNA sequences by providing repair or donor templates. Genome editing using SDN technology is categorized generally in three categories (SDN1, 2,3) [4].

Technique SDN1: sgRNA-Cas9 activity is delivered into cells and introduces a targeted DSB. DSBs repaired by NHEJ may lead to site specific random mutations. In extension to the original definition, two DSBs can be placed by delivery of sgRNA-Cas9 modules targeting different locations, resulting in deletion of the region in-between. Per definition, repair or donor templates are not used.

Technique SDN2: sgRNA-Cas9 activity together with a DNA repair template is delivered into cells. The repair template is homologous to the targeted region with exception of the pre-defined site specific mutations. sgRNA-Cas9 activity induces a targeted DSB. In the course of HDR, the repair template may be used and the desired site specific mutations are implemented at the targeted genomic locus.

Technique SDN3: sgRNA-Cas9 activity together with a repair template harbouring a cis-, intra-, or transgene is delivered into cells. CRISPR-Cas9 induces a targeted DSB. In the course of HDR, the ectopic DNA is inserted at the target site. In extension to the original definition, targeted gene insertion can also be achieved by using the repair pathway of NHEJ.

2.2 Application in plant breeding

Table 2.1 lists examples of crop trait development by CRISPR-Cas9 based genome editing.

A short overview of to date particularly discussed applications of the SDN1 technique, (i) elimination of unwanted compounds (e.g. compounds that negatively affect nutrient uptake, have allergenic potential or exhibit toxicity), (ii) increasing compounds of value (e.g. oleic acid for production of oils with high thermal stability), and (iii) engineering pathogen resistance (on recessive resistance genes [5]), is given in the study.

The SDN2 technique is especially of interest for transferring favourable functional molecular variation between cultivars or from closely related (wild) species (allele transfer) but also to engineer favourable functional variation deduced from methodological genetic screens *in vivo* or *in vitro* (e.g. directed molecular evolution of enzymes) into genomes with related sequences.

Genome editing (SDN1,2) allows targeted modification of genome sequences and complements other methods in plant breeding that generate genetic variation, like mutation breeding and transgene technology. Conventional mutation breeding programmes offer the discovery of novel, artificially induced, trait variation. The potential of SDN1 and SDN2 techniques in genome editing is linked to already present and increasing knowledge derived from basic and applied research on molecular variation underlying phenotypic trait expression as well as on knowledge on gene function and metabolic pathways in general. By using SDN1 and SDN2 techniques, the breeder directly and specifically works with the understanding of molecular variation that has been discovered to underlie phenotypes of agronomic interest. Data sets with information on genetic variation within and between species (150 Tomato Genome ReSequencing Project [6], 3000 Rice Genomes Project [7], 44 sorghum line genomes [8], 302 soybean accessions [9], 115 cucumber lines [10]), may be mined for meaningful variation of trait expression in high throughput phenotypic screens [11]. Therefore, it may be expected that additional traits (affecting, yield, quality parameters, disease resistance) may be implemented in crops using the SDN1 and SDN2 techniques.

The SDN3 technique can be used to insert cis-, intra-, or transgenes at pre-defined genomic sites. By that, it may take advantage of knowledge about regions of permissive gene expression. With this technique also gene stacking, i.e. the introduction of several genes, in close proximity is possible. This facilitates breeding programmes in that favourable new traits are not separated in successive breeding cycles and in turn can be easily introduced into further varieties/germplasm segregating as a single-locus trait.

Aside from its use in genome editing, CRISPR-Cas modules have been suggested as a basis for development of virus resistance loci in plants. Recently, proof of principle experiments have shown that CRISPR-Cas9 based expression cassettes can be used to engineer protection against different types of geminiviruses in *N. tabacum* and *A. thaliana* [12-14].

CRISPR-Cas9 technology has been shown to be transferrable to various crop plants, for example to soybean (*Glycine max*) [15], wheat (*Triticum aestivum*) [16], maize (*Zea mays*) [17], barley (*Hordeum vulgare*) [18], potato [19] and tomato [20] (*Solanum tuberosum* and *S. lycopersicum*), but also for example to tree species, like *Populus* [21] and *Citrus* [22].

2.2.1 Off-target activity

Off-target activity was also analysed in plant systems (Table 7.1 in the study). Based on the published data, specificity of CRISPR-Cas9-based genome editing in plant cells seems to be governed by the same factors as in other eukaryotic systems (in relation to spacer/protospacer interaction and PAM sequence).

Generally, for plant breeding applications, CRISPR-Cas9 specificity is of importance, however, since during plant breeding practices often several generations are passed with selection based on phenotype and/or genotype and there is the possibility of backcrosses, off-target effects are tolerable and can easily be removed, analogous to classical mutation breeding.

There are several strategies to limit off-target effects. For example, the smart selection of target sequences [23, 24], the use of paired nickases or of RNA-guided FokI nucleases [25].

2.3 Intended and unintended effects of CRISPR-Cas9 -based genome editing

The intended effect using CRISPR-Cas9 in genome editing is the targeted site specific modification of a target locus. Potential unintended effects are (i) off-target activity, (ii) retention of a CRISPR-Cas9 expression cassette transgene in resulting organisms, (iii) generation of background mutations due to transformation related procedures (cell culture passage) which are passed on to resulting organisms, and (iv) viral contamination of resulting organisms in the case of using viral vector systems.

2.4 Safety considerations of CRISPR-Cas9 -based genome editing

2.4.1 SDN1 technique

Provided that resulting plants do not carry a CRISPR-Cas9 expression cassette stably integrated in the established plant line, either because an integration did not take place during the production process, or the transgenic locus was segregated away, intended and unintended changes occurring due to the application of the SDN1 technique can be compared to those occurring during conventional mutagenesis.

2.4.1.1 Comparison of CRISPR-Cas9-based genome editing (SDN1) and conventional mutagenesis techniques in relation to mutational load and type of modifications

In comparison to conventional mutation breeding techniques (using for example gamma- or X-rays, or chemical mutagens) , the CRISPR-Cas9 SDN1 technique induces specific mutations at intended loci and potentially a smaller number of further off-target loci that can be predicted to a certain extent. This also reflects the difference in intended use of these techniques in breeding applications. Based on the available datasets today, it may be assumed that the random unintended mutational load of CRISPR-Cas9 genome

edited plants is much smaller in comparison to conventional mutation breeding methods, and the generated intended and unintended mutations are not qualitatively different.

2.4.1.2 Safety considerations in respect to CRISPR-Cas9 transgene retention, background mutations caused by transformation procedures and the use of viral vector systems

For safety considerations in respect to transgene retention, background mutations caused by transfromation procedures and the use of viral vector systems, please refer to chapter 3.4, since these safety considerations are covered in the context of rapid-cycle breeding.

2.4.2 SDN2 technique

The SDN2 technique targets specific loci to introduce mutations of *a priori* known sequence changes. For that, together with the CRISPR-Cas9 module DNA repair templates are co-transformed that are identical in sequence to the targeted locus with the exception of the intended, pre-defined sequence changes.

For the SDN2 technique, the same applies in regard to safety considerations as in regard to SDN1. Additionally, using the SDN2 technique,the repair template may be integrated as an ectopic, cisgenic locus at the genomic site with the induced DSB, as well as at other sites in the genome. Analysis of genome edited plant lines for ectopic integration of cisgenes is done by standard methods (Southern Blot, PCR based methods) and plant lines without ectopic integration events can be selected accordingly.

2.4.3 SDN3 technique

In contrast to conventionally generated cis-, intra-, and transgenic plants, the SDN3 technique is used to insert DNA at *a priori* intended loci. Safety aspects concerning impairment of endogenous genes and creation of novel reading frames can therefore be already addressed at the development phase of plant lines.

Safety aspects of of cis- and intragenic plants have been covered in comparison to transgenic plants in the study of AGES [2] and in a Scientific Opinion by EFSA [26].

2.5 Detection and identification of CRISPR-Cas9 -based genome editing

In cases where a CRISPR-Cas9 expression cassette is present in the resulting plant line, detection and identification rationale follows those of conventionally transgenic plants. CRISPR-Cas9 expression cassette sequences in combination with the genomic integration location then provide a marker for GMO detection and event-specific identification. However, it is to be expected that genome edited plant lines free of CRISPR-Cas9 transgenes will be established.

2.5.1 Detection and identification of SDN1 and SDN2 genome editing

The quality of SDN1 and SDN2 mutations do not allow conclusions on their origin

Nucleotide changes (down to single nucleotide polymorphisms (SNPs)) are detectable by standard PCR based, hybridization based or sequencing methods [27]. The induced genomic changes cannot be distinguished from naturally occurring variation or from changes derived from conventional mutagenesis (see chapter 2.7.1.1). Therefore, the presence alone of a mutation at a genomic site cannot be causally linked to it being generated by the application of CRISPR-Cas9 technology. Circumstantial evidence based on background markers may be used for identification of a genome editing event. In case a particular mutation of a genome editing event is described in combination with marker states of the background genome of the plant line in which it was generated, these in combination may be used to indicate the probability of the origin of a mutation (and therefore identification) in a sample. However, the use of the genome edited line in breeding programmes will break up linkage to background markers and therefore decrease or abolish evidence of the origin of the mutation.

2.5.2 Detection and identification of SDN3 genome editing

Detection and identification of SDN3 genome editing follow the same principle as for conventionally generated transgenic plants. For cis- and intragenic lines the detection step, i.e. the detection of distinct sequences indicating cis- or intragenic status in a general screening step, is made more labour-intensive because of sequence homology of inserted sequences to endogenous genes (cis- and intragenic plants were discussed by AGES [2]).

2.6 Aspects relating to GMO classification of CRISPR-Cas9 -based genome editing

A position statement of the ZKBS (Zentrale Kommission für die Biologische Sicherheit, Germany; established under the scope of the german gene technology act) in 2012, includes the assessment of ZFN technology [28] and in extension their assessment may be extrapolated to other DSB producing site directed nucleases (SDN). In their statement they provide conclusions on their interpretation of the term GMO in Directive 2001/18/EC in relation to SDNs.

While the SDN3 technique generates cis-, intra-, or transgenic plants falling under the EU GMO definition (Directive 2001/18/EC), there is legal uncertainty whether genome modified plants resulting from SDN1 and SDN2 techniques do so as well.

The SDN1 and SDN2 technique lead to plants with targeted introduced mutations, which are not distinguishable from genetic modifications generated by natural processes or by mutation breeding. In the

process of establishing SDN1 and SDN2 genome edited plants intermediate plants may be generated that stably integrate a CRISPR-Cas9 transgene in their genome. In sexually propagated crops, the transgene and the intended genome modification can be separated resulting in progenitors harbouring the genome modification but not the CRISPR-Cas9 transgene. Furthermore, techniques delivering sgRNA-Cas9 modules into cells without transfer of heritable, genetic material are being developed.

The ZKBS concludes that, in their scientific opinion, resulting organisms demonstrably free of genetically modified nucleic acids are not falling under the GMO definition under 2001/18/EG [28].

Directive 2001/18/EC explicitly excludes plants from its scope generated by specified methods; these are conventional mutagenesis breeding and plants generated by cell or protoplast fusion, as well as it does not consider plants generated by polyploidy induction. Plants generated by these techniques are exempted from the risk management and regulatory procedure established by Directive 2001/18/EC.

It can be concluded, therefore, that Directive 2001/18/EC implicitly states that the risks associated arising from intended and unintended mutations by the exempted techniques (mutagenesis breeding, cell culture methods, crossing between species, polyploidy-induction), are considered to be manageable outside the regulatory procedure of Directive 2001/18/EC, that is, by the breeding practices implemented by breeders. From a scientific view, the mutations – intended and unintended - generated in (cis-, intra-, and transgene free) genome edited plants are not qualitatively different from plants arising from natural mutation events or generated by breeding practises not falling under Directive 2001/18/EC. With respect to the quantity of mutations, genome editing induces a minimal number of mutation events compared to e.g. chemical mutagenesis breeding (for the latter typically in the order of 100s to 1000s mutations per individual).

2.7 Table

Table 2.1 SDN1 genome edited crop lines with track record in databases of competent authorities

Crop	Genome modification technique	Trait	Developer	Authority
Maize	CRISPR – SDN1	high amylopectin content	DuPont Pioneer	USDA/APHIS[a)] April 2016
White button mushroom	CRISPR – SDN1	non browning	Pennsylvania State University	USDA/APHIS[a)] April 2016
Wheat	TALEN – SDN1	improved disease resistance to powdery mildew	Calyxt [29]	USDA/APHIS[a)] February 2016
Maize	Meganuclease – SDN1	starch accumulation in leaf and stalk tissue	Agrivida	USDA/APHIS[a)] Novmber 2015
Rice	TALEN – SDN1	to be field tested for increased bacterial blight resistance	Iowa State University [30]	USDA/APHIS[a)] Novmber 2015
Soybean	TALEN – SDN1	high oleic acid content	Cellectis	USDA/APHIS[a)] May 2015
Maize	Meganuclease – SDN2/3?	increased photosynthetic efficiency	Benson Hill Biosystems	USDA/APHIS[a)] May 2015
Potato	TALEN – SDN1	undetectable levels of reducing sugars; reduced levels of acrylamide in processed chips[b]	Cellectis [31]	USDA/APHIS[a)] November 2014
Maize	ZFN – SDN3	reduced phytate production	Dow Agro Science [32]	USDA/APHIS[a)] March 2012

[a)]US Department of Agriculture, Animal and Plant Health Inspection Service; recorded under Regulated Letters of Enquiry (under 7 CFR part 340) (https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated)

[b)]not disclosed in letter to USDA/APHIS; see literature reference [31]

3 Accelerated breeding – rapid cycle breeding

3.1 Introduction

Accelerated breeding, also called rapid cycle breeding, is a technique to shorten the duration of breeding programmes [33]. Specifically of interest in species with long generation times, as in perennial, woody plants, it is achieved by establishing plant lines carrying transgenes that confer a dominant precocious flowering phenotype. These lines are used as crossing partners to shorten individual breeding cycles. At the end of the breeding process, individuals carrying the desired trait/trait or trait/genomic background combinations but lacking the early flowering transgene are selected for further propagation (null-segregants) [33].

Rapid cycle breeding, together with developments in marker assisted selection (MAS), offers the potential to increase the number of breeding programmes in perennials by reduction of time and cost of infrastructure [34-36].

3.2 Application in plant breeding

Plant breeding in species with long juvenile phases, such as in shrubs or trees, is a time consuming process. The juvenile phase can, for example, last up to 6 - 12 years for apple (*Malus domestica*) and pear (*Pyrus communis*) in field conditions [37]. Rapid cycle breeding could contribute to an increase in breeding programmes in species with long juvenile phases. By that, desired breeding objectives in these species, for example the introduction of disease resistance genes from related species, could be realized in a reasonable time-frame.

The research focus for applications in breeding is in woody, perennial species; the most scientific publications can be found for apple and poplar, followed by citrus. Single studies can also be found for birch, eucalyptus, pear and plum in the scientific literature (for references and an overview please refer to Table 3.1 in the study). Furthermore, there is one study published describes the accelerated breeding strategy in soybean [38].

Breeding programmes in apple (*Malus domestica;* breeding objective pyramiding of resistance genes) and plum (*Prunus domestica;* breeding objective transfer of resistance gene between cultivars, breeding for high sugar content) have been established, both with involvement of Federal Research Agencies.

3.3 Intended and unintended effects

The intended effect of rapid cycle breeding is, by using a transgenic, precocious flowering breeding partner, to shorten the duration between successive crosses.

Possible unintended effects by means of using transgenic plant lines as breeding partners may be (i) retention of the transgene in resulting organisms and (ii) generation of background mutations in the transgenic precocious flowering lines due to the performed transformation process which are passed on to resulting organisms, and (iii) in the case of using viral vector systems, viral contamination of resulting organisms.

Possible unintended effects caused by the novel combination of different genomic backgrounds due to the breeding process are not unique to or caused by application of rapid-cycle breeding and may occur as in conventional breeding programmes.

3.4 Safety considerations

Retention of transgene in resulting organism

Resulting organisms in a rapid cycle breeding programme are selected based on the desired trait/trait or trait/genomic background combination analogously to conventional breeding programmes, additionally, resulting organisms lacking the precocious flowering phenotype conferring transgene are selected. Transgenic lines generated for rapid cycle breeding are evaluated for transgenic state (insert number, location) since it is integral to an efficient breeding programme to use well characterised transgenic lines. Presence/absence of the transgene is monitored during the rapid cycle breeding process to ensure the use of appropriate breeding partners (chapter 3.2.4 in the study). PCR techniques are used to map transgene insertion sites, Southern blotting is a standard method to analyse transgene copy number.

For confirmation of transgene absence in resulting organisms, PCR techniques and Southern Blotting can be used, furthermore, it is possible to analyse transgene absence by genome sequencing using next generation technologies [39].

Background mutations caused by the transformation procedure elsewhere in genome

Experimental procedures during establishment of transgenic lines may lead to mutations elsewhere in the genome. Background mutations may be silent as well as non-silent in regard to changes in the expression of the genome. In the latter case, mutations may have beneficial or adverse effects, or may be neutral, the categorisation may change also depending on the environmental conditions. Unintended, unknown mutations similarly arise in conventional and mutation breeding as well as by tissue culture based methods.

Viral contamination

Precocious flower formation may be induced using viral vectors. Several strategies of viral elimination have been established, furthermore, viral absence in resulting organisms can be analysed via tests for viral DNA, RNA or protein epitopes [40].

3.5 Detection and identification

Intermediate plants with the precocious flowering phenotype may be transgenic plants. In that case, the transgenic locus in combination with its genomic integration location provides a marker for GM detection and event-specific identification.

Resulting organisms do not carry the precocious flowering conferring transgene and therefore cannot be detected or identified as being generated by rapid-cycle breeding by means of current detection methods.

3.6 Aspects relating to GMO classification

Intermediate plants with a stably inserted transgene fall under the GMO definition (Directive 2001/18/EC). To date, there is legal uncertainty whether plants resulting from rapid cycle breeding and lacking a transgene do so as well.

The ZKBS published a position statement, in which they conclude on an analogous breeding practise, reverse breeding, that transgene free resulting organisms, in their opinion, do not fall under the EU GMO definition [28].

4 Small RNA-directed techniques

4.1 Introduction

RNAi-based methods exploit the naturally occurring cellular RNAi machinery in order to downregulate or silence expression of target RNAs. Central to triggering RNAi are double stranded RNA (dsRNA) molecules of diverse sources [41]. In the course of the RNAi pathway, they are processed into short, single stranded RNAs and exert their function within a ribonucleo-protein complex termed RISC (RNA induced silencing complex). In a natural context, dsRNA may stem from diverse sources, and in RNAi-based methods it is delivered into cells by transgenic expression cassettes.

Expression of genes may be influenced at the post-transcriptional level (PTGS, post-transcriptional gene silencing). Depending on the particular RNAi pathway, sRNA – target recognition may also result in transcriptional gene silencing (TGS). In the former, RNA targets are cleaved or translationally repressed/destabilized, in the latter epigenetic modification (RNA-directed DNA methylation (RdDM)) is induced, resulting in silenced transcription of target loci. TGS was reported on in a study by AGES [3], and is not further addressed in this study.

RNAi pathways are further grouped based on origin and biogenesis of sRNAs and engaged members of DCL and AGO proteins into microRNA (miRNA) and small inhibitory RNA (siRNA) pathways [41-43].

4.1.1 miRNAs - siRNAs

miRNAs in plants have been shown to be involved in regulation of plant developmental processes and in biotic and abiotic stress responses [44]. They are encoded at *MIR* loci, non-protein coding nuclear genes [42]; many plant species code for ≥ 100 loci [45].

Plant siRNAs are generated from diverse sources. Common to all, and as a distinctive feature to miRNA biogenesis, siRNA pathways do not depend on single, unique siRNAs but usually dsRNA is diced into several entities (Fig. 4.2 and 4.3 in the study). siRNA pathways in plants further may involve signal amplification steps carried out by RdRPs (RNA-dependent RNA Polymerases) [41-43] which additionally to signal amplification may lead to transitive signals, i.e. secondary siRNAs different in sequence to the primary siRNAs [46].

4.2 Application of RNAi-based approaches in plant breeding

Table 4.1 (to be found in the study) lists RNAi-based transgenic crop plants present in the scientific literature; entries are selected to exemplify potential areas of application in plant breeding. Table 4.2 lists

examples of RNAi-based transgenic crops which have been developed for the market and have already been evaluated by regulatory agencies; some of these are or had been placed on the market.

The RNAi-based transgene may target plant endogenous genes, and thereby affect quality or agronomical traits as well as for example affect traits involved in abiotic and biotic stress tolerance. Among published crop plants with altered quality traits, there are examples with increased content of substances of value (f.e. amylose in wheat; [47], secondary metabolites in rapeseed or tomato; [48, 49]); furthermore example with reduced content of unwanted compounds (f.e phytate in rice [50]; immunogenic epitopes in apple [51, 52] and carrot [53]; α- and/or ω-gliadins in wheat [54-56]. Stress tolerance to drought was engineered in canola, corn and potato targeting different classes of genes [57-60]). The possibility to implement resistance against pathogens was shown f.e. in rice (*Xanthomonas oryzae* [61]). There are several examples of RNAi based transgenic crop plants that have passed regulatory approval (Table 4.2). In the EU there are two soybean lines authorized altered for increased oleic acid content. Further, recently authorized transgenic plants in the US are a potato and an apple line engineered to withstand oxidative browning after slicing or bruising. The potato line additionally is engineered for purposes of processing involving heat treatment: it does form less acrylamide upon frying.

RNAi-based applications may also target genes expressed in plant pathogens. The latter can be used to establish plants resistant to viral diseases [62] [63] [64], or, collectively termed host induced gene silencing (HIGS), to protect against insects [65] and nematodes [66-68] feeding on plants, as well as fungal and possibly bacterial diseases [69-71]. HIGS-based approaches are new developments in cultivar improvement. Recently, US-EPA (US Environmental Protection Agency) issued a registration note concerning field-testing of a maize line (MON-87411-9) engineered via a HIGS-based approach to target an essential gene of the western corn root worm (Table 4.2).

4.3 Intended and unintended effects

The intended effect of RNAi-based approaches are (i) the reduction of expression of target genes, or (ii) the reduction of the presence of RNA molecules of other origin. Target RNA may be plant endogenous RNA or may stem from pathogens interacting with plants (viral RNA, HIGS-based approaches).

Potential unintended effects are off-target effects, which may lead to unintended downregulation of endogenous plant genes, as well as of downregulation of gene expression in non-target organisms (NTO).

4.4 Safety considerations

Currently, specifics in regard to risk assessment of RNAi-based GM plants are discussed [72], at the EU level by EFSA (European Food Safety Authority).

EFSA develops guidelines for risk assessment of genetically modified organisms (GMO) based on EU regulations. These documents provide guidance on the specific provisions for submission dossiers for authorization of GM plants under Regulation (EC) No. 1829/2003 on GM food and feed or under Directive 2001/18/EC on the deliberate release into the environment. The majority of authorized GM plants internationally and in the EU are based on transgenic plants expressing one or more novel proteins, however, commercial development of RNAi-based GM plants is expected to increase due to its potential for example in engineering pest resistance or altering crop composition [73].

EFSA organized a scientific workshop “Risk assessment considerations for RNAi-based GM plants” [74, 75] in order to formulate and discuss specific features of RNAi-based GM plants. Building on that, in 2015, EFSA published a call for a “Literature review of baseline information to support the risk assessment of RNAi-based GM plants” (OC/EFSA/GMO/2015/01; OC/EFSA/GMO/2015/02). Scientific baseline data present in the scientific literature in areas relevant to (i) the molecular characterization, (ii) the food and feed risk assessment and (iii) the environmental risk assessment will be collected and assessed. In regard to these areas, EFSA formulated specific questions to be analysed. The literature review therefore will inform, if necessary, on potential future areas of research to close knowledge gaps, and will provide information on potential adaptations to the current framework of risk assessment of GM plants in regard to specifics of RNAi-based GM plants.

4.5 Detection and identification

Genomes of RNAi based GM plants contain a stably integrated transgene that in combination with its genomic integration location can be used to develop an event-specific detection method for identification. In case the transgenic construct contains elements often used in development of GMOs these can be employed for screening assays for detection purposes. Examples provide the event specific identification methods for RNAi based GM plants soybean MON 87705 and soybean DP-305423-1 listed in the GMOMETHODS database [76, 77].

4.6 Aspects relating to GMO classification

RNAi-based GM plants fall under the legal definition of GMO given in EU Directive 2001/18/EC.

4.7 Table

Table 4.1 RNAi based transgenic crops which have been evaluated by regulatory agencies and have been approved for commercial purposes or [+]agronomic evaluation

Species	Trait	Agency	Developer
	biotic stress resistance traits		
Plum (Event C5; `Honeysweet´)	Plum pox virus resistance (PPV)	USA: Determination of Non-regulated status by APHIS, USA 2007** US-FDA completed review 2009* US-EPA registration 2010 §	US Department of Agriculture (USDA) Agricultural Research Service (ARS) in cooperation with Research Institutes in Europe
Common Bean (EMBRAPA 5.1)	Bean golden mosaic virus (BGMV) resistance	Brazil: Regulatory approval for food, feed and cultivation 2011*,§§	Embrapa, Brazilian Agricultural Research Corporation
Maize [+] MON-87411-9	*Diabrotica virgifera virgifera* (Western corn rootworm (WCR)) resistance	USA: Determination of Non-regulated status by APHIS, USA 2015** US-FDA completed review 2014* US-EPA registration 2015 for agronomic evaluation (not authorised for commercial purposes) [+]	Monsanto
	quality traits		
Potato Innate™ potatoes 1st generation	impaired black spot bruise development impaired asparagine (Asn1) and reducing sugar formation (pPhL, pR1) which leads to low acrylamide content upon heat treatment (frying, baking, cooking)	USA: Determination of Non-regulated status by APHIS, USA, 2014** US-FDA completed review 2015*** for events in bold	J.R. Simplot Company, USA
Apple Arctic™ Apple Events GD743, GS784	impaired enzymatic browning of apple flesh after slicing or bruising	USA: Determination of Non-regulated status by APHIS, USA, 2015** US-FDA completed review 2015*** Canada: Health Canada: approved product for sale and growth as	Okanagan Specialty Fruits Inc, Canada

Species	Trait	Agency	Developer
		GM Food 2015 *, #	
Alfalfa KK179	Reduced lignin content which allows greater flexibility in harvest timing; high lignin content affects quality negatively	USA: Determination of Non-regulated status by APHIS, USA, 2014** US-FDA completed review for use in animal feed 2013***	Monsanto; Forage Genetics International, USA
Soybean MON 87705 (Vistive Gold™)	increased oleic acid and reduced linoleic acid content, which confers higher oxidative stability of the oil	EU: Authorisation for use as/in Food and Feed 2015 ### USA: Determination of Non-regulated status by APHIS, USA, 2011** US-FDA completed review 2011***	Monsanto
Soybean DP 305423 (Plenish Soy)	increased oleic acid and reduced linoleic acid content, which confers higher oxidative stability of the oil	EU: Authorisation for use as/in Food and Feed 2015 ### USA: Determination of Non-regulated status by APHIS, USA, 2010** US-FDA completed review 2009***	DuPont Pioneer
Tomato FlavrSavr™	Decreased cell wall breakdown which confers longer shelf life; processed tomatoes with higher serum viscosity	USA: Determination of Non-regulated status by APHIS, USA, 1992** US-FDA completed review 1994*	Calgene, USA

Listed RNAi plant lines may contain further transgenes to confer additional traits (for example herbicide resistance of MON87705), described are only traits based on an RNAi transgene. Listed RNAi plant lines may have gone through regulatory approval in further countries.

* Center for Environmental Risk Assessment (CERA) (http://www.cera-gmc.org)

** Petitions for Determination of Nonregulated Status Database, US Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS): https://www.aphis.usda.gov/biotechnology/petitions_table_pending.shtml

*** US-FDA Inventory on Biotechnology Consultations on Food from GE Plant Varieties: http://www.accessdata.fda.gov/scripts/fdcc/?set=Biocon

Health Canada, Novel Food Decisions: http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/index-eng.php

FSANZ Food Standards Code – Standard 1.5.2 – Food produced using Gene Technology https://www.comlaw.gov.au/Series/F2008B00628/Compilations

EU Register of authorised GMOs http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

§ US Environmental Protection Agency (EPA) Plant Incorporated Protectant (PIP) registrations: http://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/overview-plant-incorporated-protectants

§§ ISAAA, International Service for the Acquisition of Agri-Biotech Applications, GM Approval Database: http://www.isaaa.org/gmapprovaldatabase/default.asp

5 Abbreviations

Cas CRISPR associated

CRISPR Clustered regularly interspaced short palindromic repeats

ds double stranded

DSB double strand break

EFSA European Food Safety Agency

GM genetically modified

GMO genetically modified organism

HDR homology directed repair

HIGS host induced gene silencing

miRNA micro RNA

NHEJ non-homologous end joining

nt nucleotide

NTWG New Techniques Working Group

PCR polymerase chain reaction

PTGS post-transcriptional gene silencing

SDN site directed nuclease

RISC RNA induced silencing complex

RNAi RNA interference

sgRNA single guide RNA

siRNA small inhibitory RNA

ss single strand

TGS transcriptional gene silencing

ZFN zinc finger nuclease

ZKBS Zentrale Kommission für Biologische Sicherheit/Central Commission for biological Safety

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