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New developments in green biotechnology – an inventory for RIVM

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1. Executive summary

This study is an exploration of developments in "green" (plant) biotechnology for the purpose of policy development and updating risk assessment of GMOs and biotechnology in general in the Netherlands. The study is based on a literature review and interviews with stakeholders. The developments encompass both novel techniques such as genome editing and new concepts or applications for already established techniques such as plant transformation (transgenic plants). Intended and unintended effects are discussed, but risk assessment and regulatory issues are not, since they are out of the scope of this inventory.

The overview of developments in plant biotechnology is presented as a listing of techniques and/or concepts (see Table 1):

- Genome editing generates mutations in specifically targeted genomic sequences, such as genes or gene promoters. This can be performed by introducing sequence-specific oligo nucleotides into plant cells (protoplasts) in oligo-directed mutagenesis (ODM). Alternatively, sequence-specific nucleases (SSNs) are used that make double-strand breaks followed by repair (non-homologous end-joining NHEJ) leading to local changes in the sequence. The presently most notable SSN is CRISPR-Cas. The SSNs can also be used for introduction of larger sequences, including genes or alleles, by using a homologous recombination (HR) repair mechanism.
- Alternative uses of classical transformation technology: Using transgenic plant lines without the transgene becoming part of the final plant product can for instance be applied to speed up breeding in crops with long generation times, e.g. trees that only start flowering a few years after seed germination. Transgenic lines that start flowering in their first year are used to speed up a crossing scheme to introduce new traits into elite plant material. By selecting against the transgene during the final crossing steps, a plant product (variety) is obtained that does not contain the transgene anymore. These are also called "null segregants". Such methods can also be applied in various forms in hybrid variety breeding. Cisgenesis and intragenesis, which are concepts of classical transformation using only genes from cross-compatible species are also considered in this study.
- RNAi (RNA interference) targets specific genomic sequences as in genome editing, but in this case genes are silenced using RNA interference constructs. This can be applied in two ways: (I) by generating small RNAs directed at specific mRNAs inhibiting their translation into proteins (post-transcriptional gene silencing (PTGS)); or (II) by generating small RNAs directed at specific gene promoters effecting DNA methylation in turn leading to silencing of gene transcription of mRNA (transcriptional gene silencing (TGS), also designated as RNA-directed DNA methylation (RdDM).
- Synthetic biology involves creating plant parts by design, such as artificial chromosomes. It is not always clearly defined. In this inventory, we have discussed novel metabolic pathways based on classical plant transformation in this category.
- We briefly discuss remaining categories of external compounds directing gene expression and an additional development in grafted plants.

There are many cross-connections among these techniques and concepts that are shown in an alternative scheme in Table 2. The scheme is based on the way transgenic constructs are used: plants with stable transgene insertion(s) into their genome (e.g. RNAi, particularly PTGS), with initial transgene insertion, but followed by subsequent removal of the transgenic constructs (e.g. the early flowering), and transient expression (no insertion of transgenes into the genome) or introducing only (ribo)proteins or RNA into the plant cell for changing traits by genome editing (DNA-free genome editing).

Genome editing and RNAi target existing ("native") plant genes. An exception is the application of genome editing by homologous recombination (HR) using "foreign" sequences as template. ODM can produce gene knock-outs and small changes of one amino acid. Genome editing using SSNs following the non-homologous end joining (NHEJ) pathway will mainly produce gene knock-outs and genome editing by HR enables to produce precise changes, from small sequence changes up to complete allelic or (novel) gene sequences. With RNAi, gene knock-downs are produced by silencing the expression of native genes, but as opposed to genome editing knock-outs, they will inherit dominantly, as long as the silencing (RNAi-) construct is present in the plant line. This means that the trait is always effective (also at a heterozygous state), as opposed to gene knock-outs that are only expressed in a homozygous (recessive

state), i.e. with all alleles of a gene being defective in expression. Silencing using RNAi will often not be absolute, but this may be advantageous for traits where complete knocking out of the gene function has undesirable side effects. Among these traits, an interesting example is resistance to insects or pathogens through "cross-kingdom" RNAi, i.e. post-transcriptional gene silencing (PTGS) by a transgenic construct targeting gene sequences of the pathogen or pest, also called host-induced gene silencing (HIGS). It represents a novel type of trait, though exchange of small RNAs between organisms is an existing phenomenon. Enabling technologies of next generation sequencing (NGS) and bioinformatics will increase the ability to find target organism-specific sequences. These enabling technologies are also helpful with the other techniques, for example by identifying genes interesting for changing expression by genome editing or RNAi, or for targeted introduction by e.g. SSN - HR.

As in classical plant transformation, in the new developments of green biotechnology, intended and unintended effects will largely depend on the type of new trait introduced or generated and so will vary from case to case. Most techniques enable changing a wide variety of traits, for instance, abiotic stress tolerances (e.g. for drought or salt), disease resistances (e.g. against bacteria or fungi) or metabolic changes underlying product quality.

Depending on the specific SSN variant, off-target mutations (unintended modifications at other chromosomal locations) may occur to a certain extent. For CRISPR-Cas9, improvements have been made in order to reduce the off-target effects. In addition, such effects could now be identified more efficiently using the enabling technologies of NGS and bioinformatics. Likewise with RNAi, off-target silencing also appears to be possible. Nevertheless, genome editing (and for that matter, gene silencing) is expected to be basically more precise than "classical" mutagenesis as it is targeted at specific sequences in the genome. "Classical" mutagenesis induces multiple random mutations in the genome among which the trait of interest needs to be selected and subsequent plant breeding is used to apply extensive phenotypic and increasingly genotypic testing for selecting elite materials for commercialization.

Drivers behind many of the new techniques or concepts discussed in this report appear to be of a dualistic nature. From a technical point of view, they primarily aim at improving the efficiency and/or precision of breeding, but the possibility of simplifying regulatory oversight is also of relevance, in that they mostly avoid the presence of transgenes in the final plant product (cf. Table 2). In particular examples, such as early flowering for faster crossing schemes, removing the transgenic construct is also an essential part of the technique as the expression of genes that are present on the transgenic construct is unwanted in the final plant product. As a result of both drivers, unintended effects associated with plant transformation will be minimized. For SSN-based genome editing techniques there is even an alternative to avoid inserting a transgenic construct into the genome: employing transient expression of the construct encoding the SSN in protoplasts or introducing mRNAs coding for the SSN proteins or the SSN proteins and/or gRNAs directly into protoplasts ("DNA-free" genome editing). For the "null segregant" approaches, including those in RNAi (e.g. RdDM), an alternative is introducing expression constructs through virus vectors that will not end up in the progeny as they are not transmitted during seed formation.

Predictions on future developments in green biotechnology are accompanied by uncertainty or can be incomplete, as exemplified by the recent rise of CRISPR-Cas, which was unknown as a genome editing tool in plants just five years ago. As already seen in classical plant transformation (transgenic plants), promising applications need to go through the stringent process - basic to all innovations - of achieving commercial viability, including competition with alternative approaches. For instance, amongst others, "cross-kingdom" RNAi may have to compete with alternative modes of application using sprays containing the interfering RNAs. A practical problem with predicting the likelihood of development into commercial products is the barrier perceived in their regulatory status as a GM or not. The costs and/or uncertainty around the consequences of regulation are perceived as such a burden that breeders will likely not use novel technologies commercially when they would fall under present GM regulation or as long as there is uncertainty about this in the EU. In an attempt to be still informative, Table 3 presents an overview of the techniques with an attempt to depict the horizon of applications, disregarding the expected impact of regulation in the EU as far as possible. The most likely techniques to show strong developments in the near future are the genome editing techniques, in particular CRISPR-Cas9. The HR variant could be most precise and versatile but is still technically demanding in plants. The "null segregant" concepts are promising for accelerating the breeding process and for several processes underlying production of hybrid seed varieties. Some RNAi applications are already on the market outside

of the EU and others may become relevant in the near future, e.g. "cross-kingdom" RNAi (HIGS) with an insect example recently deregulated in the US. But others, e.g. transcriptional gene silencing TGS (based on RNA-dependent DNA methylation RdDM), at least in their variant of the end product not containing the transgenic silencing construct, are still awaiting effective examples. Applications in metabolic engineering may become relevant soon, particularly in bio-fortification and bio-based economy, and for producing pharmaceuticals.

2. Introduction

This study entails an exploration of developments in "green" biotechnology, alongside other such inventories for the "white" (industrial) and "red" (medicinal) biotechnology executed by other research teams, for the purpose of policy development and updating risk assessment of GMOs and products of biotechnology in general in the Netherlands. The development that drew most attention in plant biotechnology in recent years has been the introduction of various novel technologies aiming at improving the precision of introduced changes in the plant genome and/or the efficiency of plant breeding. At the same time, some of these technologies raised questions as to how to define their products with regard to the existing definitions of a genetically modified organism (see the list in Lusser *et al.* 2012). These technologies comprised new tools, such as targeted genome editing, as well as new concepts, such as the use of transformed plants to improve the efficiency of the breeding process without transgene(s) being present in the final plant product. However, the ongoing expansion of plant biotechnology covers more than these techniques. There are also interesting developments in "classical" genetic modification (plant transformation), such as the application of RNAi (RNA interference) for the control of pests and pathogens. In addition, enabling technologies, particularly a rapid succession of generations of DNA sequencing technology combined with bioinformatics, are quickly expanding the knowledge of plant genomes, which in turn helps in refining the technologies modifying genomes. In addition, the end products of new technologies, such as genome editing[1], can be screened more extensively for the presence of any off-target effects. Moreover, sequencing opens up new ways of screening for useful genetic variation, which drives technological developments regarding how to make use of such variation as quickly and effectively as possible.

For the purposes of this inventory, we address techniques and concepts aimed at changing genomes and gene expression in higher (crop) plants in a heritable fashion. We will also briefly mention other methods affecting gene expression, e.g. application of various types of non-coding RNA without transformation, but only for comparison to similar processes enabled by genomic changes. Enabling technologies will be mentioned in so far as they may play a role as driver in the developments that form the primary subject of this inventory. We will describe most extensively the techniques for applications that will be found to be probably closest to marketable plant products. We will discuss the intended and unintended effects reported for the respective techniques, but risk assessment and legal issues are out of the scope of this inventory. Nevertheless, regulatory issues will be mentioned as they are often cited as barriers to commercial application of techniques. Thus, for each technique or concept, we will give (I) a technical description; (II) an overview of host effects, intended and unintended; (III) areas of application; (IV) barriers and drivers for further development of the technique/concept in the EU; (V) the horizon, i.e. what products are to be expected in the near future in the EU.

[1] Some authors prefer "gene editing" as the edits concern particular sites and not the entire genome

3. Method description and list of techniques

This exploration encompasses in the first place a literature review. We scanned the primary scientific literature using terms for the new technologies (see Table 1) in combinations with (un)intended or off-target effects, and additionally consulted review journals addressing plant biotechnology for obtaining an overview of any new developments. Next to this, we consulted plant breeding colleagues and companies. For the consultation of companies, we used a list of techniques/developments possibly relevant for product development and asked each of them to what extent they would lead to marketable products in the short and medium term. We also asked about drivers and barriers for each of the techniques and whether they foresee any other new development that was not yet on the list. The list of techniques and developments as used in the interviews and the interview questions are in the annex. Due to time limitations, we interviewed two sector organizations, Plantum and HollandBio, which in turn consulted their members, among which large companies and SMEs in vegetable, arable and ornamental crops, and one plant technology developer, KeyGene. The interview results were used anonymously in this report.

The focus of this inventory was on higher (crop) plants. The highly diverse algae, including the green algae that are most closely related to higher plants, are nowadays receiving more attention for bio-based economy purposes, but they were not part of this study. Also (plant) pest control through genetic modification of the target (pest) organisms themselves was outside the scope of this study.

Table 1. *List of new techniques and/or developments in plant ("green") biotechnology*.

Theme	Techniques	Mode of action/examples	Special
Genome editing	Oligo-directed mutagenesis (ODM)	• inducing site-specific changes in the DNA using oligonucleotides with mismatches as template: indels, nucleotide substitutions	
Genome editing	Sequence-specific nucleases (SSNs): non-homologous end joining (NHEJ)	• inducing site-specific double strand breaks (DSBs) resulting in homologous-independent DNA repair by NHEJ using SSNs (ZFN, Meganuclease, TALENS, CRISPR-Cas9): indels, nucleotide substitutions	variant: "DNA-free" genome editing by transient expression or delivery of (ribonucleo)protein or mRNA in cells
Genome editing	Sequence-specific nucleases (SSNs): homologous recombination (HR)	• inducing site-specific double strand breaks (DSBs) resulting in homologous-driven repair by HR using SSNs: targeted gene insertion, allele replacement	
Plant transformation (new variants)	Plant transformation with transgenes not in end product	• Induced early flowering for accelerated breeding in fruit trees; • Suppression of meiotic recombination (reverse breeding), maintainer lines, or haploid inducers for hybrid breeding; • transgenic rootstocks	
Plant transformation (new variants)	Plant transformation introducing genes from cross-compatible species	• Cisgenesis: introduction of genes from same or cross-compatible species • Intragenesis: introduction of novel combinations of genes & promoters from same or cross-compatible species	
RNAi	Post-transcriptional gene silencing (PTGS)	• overexpression of gene-derived inverted repeats (dsRNA) for silencing gene expression: degradation of mRNA or translational repression directed by siRNAs or miRNAs	"Cross-kingdom RNAi": RNAi against pests and pathogens (host induced gene silencing HIGS) Special variants using transformed rootstock to deliver siRNA to non-GM scion

RNAi	Transcriptional gene silencing (TGS)	• gene silencing by promoter methylation induced by dsRNA from transgene subsequently removed from end product (RdDM)	Special variants: Induced using virus not carried over through meiosis. Mobile small RNAs moving between shoot & root (grafted plants, possibility of regenerating epigenetically changed plant from non-GM part)
Synthetic biology		• Synthetic chromosomes • Site-specific activators/repressors (targeted activation or repression of gene expression by artificial factors affecting transcription or inducing epigenetic changes using combinations of active domains with SSN-derived DNA-binding domains) • Introduction of novel pathways; "plant as factory" (pharmaceuticals/antibodies) • Adapting CRISPR-Cas9 to targeting plant DNA viruses	

Method of delivery	Modifying gene expression without transformation	• Gene silencing by application of dsRNA:
		  ○ to plant (parts);
		  ○ to insects/pathogens
		• Agro-inoculation, VIGS
		• “DNA-free” genome editing (by SSN delivery as (ribonucleo)protein, mRNA or transient expression of SSN-genes in cells
			Special variant: early flowering by promoting or silencing gene expression through virus vector not transmitted to progeny

Other		• regenerating species hybrid from graft junction	

4. Techniques

4.1. Genome editing using oligonucleotide-directed mutagenesis (ODM)

Technical description

ODM introduces specific mutations at defined locations in the plant genome. For ODM synthetic oligonucleotides homologous to the target DNA, but containing mismatches, are introduced into plant cells. The mismatches in base pairing between the single-stranded oligonucleotide and target DNA are corrected by the plant's native repair mechanism, resulting in point mutations in the targeted DNA, which can be nucleotide substitutions or small indels, leading to an altered protein coding sequence, or a premature translational stop. The synthetic oligo's consist of both DNA and modified nucleotides or other end-protective chemistries. These modifications prevent the oligonucleotides from undergoing recombination (i.e., being incorporated into the genome), while maintaining the ability to act as a mutagen (Sauer *et al.* 2016). Once the correction process is completed, the oligos are degraded. In *in vitro* assays using "cell-free" extracts and various pure enzymes, it was demonstrated that the oligos are stable for sufficient time to direct gene correction and then are quickly degraded (Gocal *et al.* 2015).

Host effects

Genome editing by ODM leads to variants of native genes or knock-out mutants. As effective ODM requires long (>40 nt) oligos, (which are aside from the intended mismatch(es) fully complementary to the target sequence), off-target mutations (the unintended modifications at other chromosomal locations) are not expected. Information about any ODM off-target effects is however limited. Cole-Strauss *et al.* (1996) and Xiang *et al.* (1997) demonstrated the specificity of ODM by showing that when targeting the ß-globin locus, closely related homologous globin gene sequences remained unaltered. We have found no other studies studying potential off-target effects of ODM.

Application areas

Because the efficiency of gene modification by ODM is low, to date all published examples using ODM in plants aimed at an efficiently selectable, herbicide-tolerant (HT) phenotype. In maize, canola, and oilseed rape plants, tolerance to imidazolinone herbicides has been engineered through targeted mutagenesis of the endogenous acetolactate synthase (ALS) gene, also known as the acetohydroxy acid synthase (AHAS) gene (Zhu *et al.* 2000). KeyGene reports that they have increased the efficiency of their system KeyBase up to about 1%, which would enable generating mutations in non-selectable types of traits, significantly broadening the usage of the technique.

Barriers and drivers

The requirement of specific tissue culture technology (protoplast technology; biolistic delivery) remains a barrier to a widespread application of this technique. Successful regeneration of plants from protoplasts is only applicable to a limited number of crop species (but including important crops such as tomato, potato and lettuce) and success rates are genotype-dependent (Eeckhaut *et al.* 2013). Targeting genes that are non-selectable during the ODM process and plant regeneration require high repair efficiencies, which are difficult to realize. A driver is that it represents a precise mutagenesis method that does not involve the use of transgenic constructs.

Horizon

Two companies, Cibus and KeyGene (http://www.keygene.com/products-tech/keybase/), are known to work on development and application of ODM for genome editing in plants. Cibus has produced herbicide-tolerant oilseed rape using ODM (which they call 'Rapid Trait Development System', RTDS), which is commercialized in the United States and Canada (http://cibus.com/press/press031814.php). Interviewees indicated that ODM could be attractive as no transgenic constructs are introduced and

therefore, it could be seen as the genome editing method farthest away from classical plant transformation. Competent authorities in six EU member states (IE, UK, ES, DE, FI and SE) have indicated that they do not consider (specific cases of) ODM to fall under GM regulation by Directive 2001/18/EC. Thus, Cibus performed field trials in the UK and SE, apparently without regulatory oversight, but has stopped these activities awaiting the legal analysis by the European Commission (Abbott 2015). When ODM would be considered as falling under the Directive 2001/18/EC, this would be expected to be prohibitive for commercial applications.

Increased gene targeting efficiencies will allow modification of other types of traits, such as disease resistances and product quality improvements, for which selection in tissue culture systems is not possible.

4.2. Genome editing using sequence-specific nucleases (SSNs): non-homologous end joining (NHEJ pathway)

Technical description

Using SSN (sequence-specific nuclease) technology followed by non-homologous end joining (NHEJ)-mediated repair, any gene of interest can be stably knocked out or mutated. Zinc Finger Nucleases (ZFNs), Meganucleases, TALENs and CRISPR-Cas are examples of SSNs. SSNs are also called site-directed nucleases (SDNs). SSNs bind to a predefined target DNA sequence at which location they induce a double-strand break (DSB). Repair of the DSB by the native NHEJ pathway often results in a small insertion or deletion (indel) at the target site. Less frequently larger (20-200bp) deletions are induced. The indels are often bi- (or multi-) allelic, i.e., all copies of the gene are mutated in one regenerated plant. With the simultaneous use of two or more SSNs, multiple genes may be targeted at the same time. This is especially effective with CRISPR-Cas. The SSN constructs used are not required anymore after the modifications have been introduced. In case the constructs were incorporated as transgenic sequences they may be removed from the modified plant e.g. by segregation after crossing. Genome editing using SSNs, and especially using CRISPR-Cas, in plants has extensively being reviewed recently (see e.g. Fichtner *et al.* 2014; Puchta and Fauser 2014; Chen and Gao 2014; Bortesi and Fischer 2015; Osakabe and Osakabe 2015).

Host effects

Application of SSNs without a repair template can induce small indels in coding sequences resulting in premature stop of translation (knock-out mutants) or in translation into proteins with an altered amino acid sequence (amino acid substitutions or deletions of a few amino acids). If SSNs are targeted to the promoter sequence of a gene, the removal of specific promoter elements may result in gene variants with a changed gene expression pattern (Li *et al.* 2012). The application of a combination of SSNs can result in chromosomal rearrangements, such as deletions (Zhou *et al.* 2014), inversions, duplications (Lee *et al.* 2012) (up to a few megabasepairs) and translocations (Blasco *et al.* 2014; Choi and Meyerson 2014, Puchta and Fauser 2014). This may affect gene function, but may also have an impact on meiotic recombination (Rieseberg 2001). Off-target effects (unintended modifications at other chromosomal locations) can play a role when applying genome editing using SSNs. For TALENs off-target effects are relatively rare and depend on the number of repeats used (each repeat binds specifically to a single nucleotide). For both ZFNs and CRISPR-Cas higher frequencies of off-target effects have been reported (Fichtner *et al.* 2014). With CRISPR-Cas9, newly developed Cas9-variants with improved binding of target or non-target DNA strand to the Cas9-protein (eCas9 (Slaymaker *et al.* 2016); Cas9-HF1 (Hifi, Kleinstiver *et al.* 2016)) will reduce off-target effects. Also the use of orthologous Cas9s (from different bacterial species) that require longer PAM-sequences (e.g. -NNAGAAW for Cas9 of *Streptococcus thermophilus* instead of -NGG for Cas9 of *Streptococcus pyogenes*) results in reduced off-target activity (Ran *et al.* 2015). The search for the system with the least off-target effects is partly driven by potential applications in human patients, but plant biotechnology will benefit from these developments as well.

Application areas

SSNs without a repair template are mainly used to produce knock-out mutants and have already been applied to change oil composition by biochemical pathway engineering in oil crops (in soybean; Haun *et al.* 2014) and to achieve disease resistance by knocking out disease susceptibility (S) genes (in wheat; Wang *et al.* 2014). SSNs have also been used in rice to remove a specific element of an S gene promoter to prevent upregulation by pathogen-derived effector molecules, which led to bacterial leaf blight resistance while the gene remains functional for vital functions in the plant (Li *et al.* 2012). These are examples of how (novel) traits may be engineered by the removal or deletion of genes or elements, next to the insertion of new genes or alleles (which is the main strategy used with SSN (HR), as described in section 4.3).

Barriers and drivers

One important driver of development in this technology is the discovery of CRISPR-Cas. Other drivers are increasing high throughput sequencing potential and advancements in bioinformatics, which promotes

the discovery of gene functions, and which are also powerful tools for genome-wide screening of the final products for absence of SSN-DNA sequences and potential off-target mutations (Kim *et al.* 2015). The latter requires the availability of reference genome sequences, which are still of insufficient quality for most crop species (Feuillet *et al.* 2011). Barrier is off-target activity (but see above under "Host effects" for improvements).

Interviews confirmed that CRISPR-Cas is expected to expand enormously in the coming years, as it is the most easily applicable method of genome editing, also within the reach of small companies with limited resources for laboratory facilities. Intellectual property issues could be a barrier: the present legal dispute in the US about who can rightfully claim to be patent holder of the technique may create uncertainty around its commercial use.

Horizon

Currently, CRISPR-Cas is surpassing other SSNs as method of choice, which was confirmed in interviews. As reported for ODM, commercial applications will be dependent on decisions about regulatory oversight in the EU. Pioneer's waxy maize developed by CRISPR-Cas was very recently considered not to be regulated by USDA-APHIS in the US (https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/15-352-01_air_response_signed.pdf).

A recent publication describes the option of avoiding transformation of the plant's genome, by transient expression of SSN genes in protoplasts followed by regeneration and testing for non-integration of the SSN construct (Clasen *et al.* 2015). The "DNA-free" use of CRISPR-Cas9 as preassembled ribonucleoprotein, rather than as DNA construct, for inducing DSBs has also recently been described for gene targeting in lettuce (Woo et al., 2015). A barrier for these variants is the availability of a protocol for regeneration of plants from protoplasts (like in ODM, section 4.1), which is only applicable in a limited number of crop species.

4.3. Genome editing using sequence-specific nucleases (SSNs): homologous recombination (HR) pathway

Technical description

In an alternative SSN technology, the SSN-induced double-strand break (DSB) is precisely repaired by the native homologous recombination (HR) pathway on the basis of a DNA repair template supplied to the cell that is (partly) homologous to the target site. Using this homology-driven repair (HDR) approach, native gene sequences can be replaced by homologous sequences with small modifications. Also completely new DNA sequences can be incorporated at a predefined chromosomal target site. The application of SSN technology aimed at HDR is also referred to as gene targeting (GT) (Osakabe and Osakabe 2015; Puchta and Fauser, 2013). HDR is a very inefficient process in plants and requires selectable markers to effectively recover GT events (Endo and Toki 2014).

Host effects

The host effects are dependent on the type of insert and range from changes in gene expression level and pattern (in case regulatory elements have been replaced) or changed functionality of native genes to complete new gene functions in case novel coding sequences are introduced. As unintended effect, the SSN-induced DSB can result in indels instead of gene correction or replacement, or additional indels at the same locus in homologous chromosomes in case the NHEJ-repair pathway is activated, which is more efficient in plants than the intended HR repair pathway. Similarly as described for genome editing using SSNs and NHEJ pathway, potential off-target indels caused by NHEJ-repair of DSBs at off-target sites must be taken into account also here.

Application areas

There are only a few reports describing the application of HDR for genome editing in plants. In maize and soybean (Svitashev *et al.* 2015; Li *et al.* 2015) the acetolactate synthase (ALS) gene was targeted using CRISPR-Cas9 and modified using a DNA repair template containing several nucleotide changes compared to the native sequence, thereby providing chlorsulfuron herbicide resistance. In another example using ZFNs and HDR, insertional disruption of the inositol-1,3,4,5,6-pentakisphosphate 2-kinase (IPK1) gene in maize was achieved (Shukla *et al.* 2009). IPK1 catalyses the final step in phytate (an anti-nutritional component sequestering phosphate in feed grains) biosynthesis in maize. Because the IPK1-gene is disrupted by a transgenic phosphinotricin acetyltransferase gene sequence insert (which mediates tolerance to the herbicide agent phosphinotricin (glufosinate) and is used for selection of HDR integration events), the maize plants with a reduced phytate trait still contain foreign sequences in the end product. HDR-mediated gene integration has also been used for sequential stacking genes into a so-called 'safe harbour' locus, which is a chromosomal location where genes can integrate and function in a predictable manner (Ainley *et al.* 2013). An advantage of gene stacking at a single locus is that during subsequent crossings the stacked transgenes segregate as a single locus, which would simplify breeding of plant lines with stacked transgenes (Nandy *et al.* 2015; Srivastava and Thomson 2016). There is a clear interest in stacking of transgenes. For example, Monsanto and Dow AgroSciences collaborated in the production of maize, cotton and soybean lines in which transgenes have been stacked, and commercialized these lines as SmartStax™. The largest combination to date is eight genes coding for HT and insect resistances in SmartStax™ maize, which was launched in the USA and Canada in 2010 (James 2014). These transgenes were stacked by traditional crossing.

A specific application of SSNs and HR repair described recently uses CRISPR-Cas for a mutagenic chain reaction to convert heterozygous mutations to homozygous ones (Gantz & Bier 2015). This system, called "gene drive", generated efficiently homozygous mutations in the malaria vector mosquito *Anopheles stephensi* (Gantz *et al.* 2015) and to a lesser extent in *Drosophila* fruit flies (Gantz & Bier 2015). Effective gene drives require a combination of an efficient HDR mechanism, a short generation time and genetic mixing in a population. Because in plants HDR is not efficient and so requires a strong positive selection for gene targeting events, CRISPR-Cas-mediated gene drives are as yet not expected to be effective in plants.

Barriers and drivers

The use of homologous recombination to precisely modify plant genomes has been challenging, due to the lack of efficient HR methods in plants. Current examples of genome editing by HR therefore rely on the use of selectable transgenes. Improvements, such as the use of geminivirus replicons for delivery of the SSN-coding sequences and the DNA repair template may overcome the efficiency barrier (Čermák *et al.* 2015) and drive genome editing by HR towards commercial applications.

Horizon

Precision breeding by allele replacement, replacing poor alleles by beneficial ones, including cisgenic applications (see section 4.5), promoter replacements, and stacking transgenes at a single chromosomal locus could be interesting future applications. However, commercial applications are not expected in the near future as HR is still technically challenging. The interviews indicated that this variant could be seen as quite similar to classical plant transformation (cf. EFSA's analysis of ZFN3 (SDN3, SSN3), EFSA GMO Panel 2012b) and therefore, regulatory barriers were envisaged in the EU.

4.4. Plant transformation with transgenes not in end product

Technical description

This category is a container concept rather than a technique, in which "classical" transgenic plant lines are being used in various ways to enable more efficient and faster breeding or plant (hybrid) variety production, but in which the end products do not contain the transgene any longer. In most cases, it may also be referred to as the use of "null segregants" (Camacho *et al.* 2014). The element of the use of transformation without the transgene ending up in the final plant product is actually also applicable in the genome editing techniques based on SSNs and a variant of TGS (RdDM), but these techniques are discussed in separate sections 4.2-4.3 and 4.7, respectively, because of their specific nature.

Host effects

The category covers a wide variety of approaches, which have in common that no transgene is present in the final plant products. This concept is mainly applied to facilitate and speed up plant breeding and thus, the transgene used for that purpose can or even needs to be removed by subsequent steps of crossing and screening for absence of the transgene to achieve an optimal result. Any unintended effects in the genome away from the transgene in the transgenic line will be minimal as repeated backcrosses to obtain elite material (varieties) are used and thus only a small part of the transgenic line's genome remains present in the final product (see further under section 4.5).

A special variant is the use of transgenic rootstocks. In this case, products of the scion are free of transgenes, but gene expression and (heritable) epigenetic state in the scion may be influenced through signalling pathways from the rootstock, e.g. through the transport of small RNAs (see RNAi sections 4.6 and 4.7). In addition, genetic exchanges have been reported to occur at the graft junction (see last Techniques section 4.10).

Application areas

There is a wide array of applications in which this strategy may be implemented. Examples are:

Early flowering: perennial woody crops, such as fruit trees, have long generation times and therefore lengthy breeding cycles. This can be sped up using backcrossing schemes involving lines that are early flowering by overexpressing exogenous flowering genes, such as *BpMADS4* in apple (Flachowsky *et al.* 2011) and *PtFT1* in *Eucalyptus* (Klocko *et al.* 2015) or in plum (Srinivasan *et al.* 2012), the latter called "FasTracking" (Yao 2011). In apple, proof of concept was demonstrated by combining a fire blight resistance from a wild relative (*Malus fusca*) with the new early flowering line followed by further crossing with another cultivated line containing additional disease resistances, leading to seedlings having all resistances combined within three years (Flachowsky *et al.* 2011).

Hybrid seed production: Production of doubled haploid lines that can be used as parents for hybrid seed production (and in breeding research, e.g. mapping, Van de Wiel *et al.* 2010) can be sped up by inducing haploids using a parental line with a mutated centromere-specific histone CENH3 (centromere-mediated chromosome elimination CCE). When the *cenh3* mutant is crossed to another plant, the chromosomes from the mutant are selectively lost, leaving a haploid progeny, some of which may turn into fertile doubled haploids through irregular non-reduction during meiosis. As this *CENH3* mutation is lethal, mutant lines can be maintained by introducing a transgenic rescue construct containing a variant of wild type *CENH3*. The chromosome elimination process, which also leads to loss of the transgene in the progeny, is thought to occur through unequal interactions with the mitotic spindle at the centromeres (Ravi and Chan 2010).

Hybrid seed production: Pioneer is using a transgenic maintainer line in the propagation of the male-sterile female parental line in their hybrid production system "SPT". The transgenic construct encodes a protein (MS45) that compensates the mutant version causing the male sterility of the female parent line. The transgenic construct is present in a hemizygous state and therefore is inherited in half of the pollen produced by the maintainer. The construct also encodes an α–amylase (ZM-AA1) that renders the pollen infertile and so only the pollen without the transgenic construct produce offspring that can be used as female parent in subsequent hybrid seed production. To ensure that the seeds are completely free of the

transgene, the construct contains a third gene coding for a fluorescent marker enabling to check all seeds in a non-destructive manner (Wu *et al.* 2016).

Hybrid seed production: Rijk Zwaan developed a concept for making homozygous parental lines from a superior heterozygous plant by using a transgenic or mutant line silenced in meiosis, called reverse breeding (Dirks *et al.* 2009; Wijnker *et al.* 2012).

GM rootstocks can be used for a wide array of applications, such as resistance to soil-borne diseases and pests and improved rooting ability.

Barriers and drivers

Almost all techniques (except for GM rootstocks) have a common driver in that they increase the speed of breeding and/or the efficiency of hybrid seed production.

Horizon

Hybrid seed production is being developed or searched for in many crop species. Currently, Pioneer is testing the SPT technology in rice and wheat (still in experimental phases (Pioneer 2016)). This is a potentially huge market as rice and wheat are still mostly sold as inbred seeds. The interviews confirmed that generally, among all methods to accelerate breeding, the ones improving hybrid variety breeding or enabling it in crops where this is not yet feasible, are highly interesting for breeders. For instance, Pioneer also developed a transgenic hybrid seed production system using transcriptional gene silencing (see RNAi – TGS section 4.7). As mentioned under the genome editing techniques, regulatory status affects the likelihood of commercialization in the EU. The USDA decided that the F1 hybrid varieties produced using such a system are outside the scope of regulation in the US, provided that the transgene is absent in all seeds (Camacho *et al.* 2014).

Early flowering was recently shown to be achievable using a virus vector containing a construct expressing an *Arabidopsis Flowering Locus T* gene and silencing the *Terminal Flowering 1* gene in apple (Yamagishi *et al.* 2014). The virus used is not seed-transmissible; thus, the trait is achieved without genetic modification of the apple genome (cf. similar applications in RNAi section 4.7). When the virus is applied to cotyledons, the seedling will be induced to flower, so the approach is versatile as for any seed in any generation it can be decided to induce early flowering or not. Additional possibilities with grafted plants are mentioned under RNAi sections 4.6 and 4.7.

4.5. Plant transformation introducing genes from cross-compatible species

Technical description

The central tenet in this concept is the use of sequences from cross-compatible species (also referred to as the primary and secondary gene pool (i.e., varieties and landraces, and wild species, respectively) in the context of conventional crossing methods). Two variants are discerned: cisgenesis and intragenesis (Holme *et al.* 2013). Cisgenesis involves transformation with genes from cross-compatible species in their native state, i.e. with their own promoters and introns (Jacobsen & Schouten 2007). In addition, the use of additional genes for selecting transformants should be avoided or such selection genes should be removed by inducible site-specific recombination methods (Schaart *et al.* 2011). Intragenesis likewise involves the use of genes (sequences) from cross-compatible species and marker-free transformation, but the gene sequences are recombined with e.g. other promoters or in other orientations, in order to achieve novel traits (Rommens *et al.* 2007).

Host effects

With cisgenesis, the normal effects of the genes are expected as when they would be introgressed through backcrossing. With intragenesis, the effects depend on the specific construct as with transgenesis. An already established variant is represented by constructs silencing specific genes (see under RNAi section 4.6). Other possible variants are increasing or ectopic expression of specific genes by careful choice of alternative promoters, up to rewiring of developmental pathways. Unintended effects of intragenesis will mainly depend on the type of novel trait designed (EFSA GMO Panel 2012a).

Any possible unintended effect relating to standard plant transformation (e.g. related to random insertion of T-DNA into the genome) will be similar for cis- as well as intragenesis (and transgenesis) (cf. EFSA GMO Panel 2012a; Parrott et al 2010; Ladics *et al.* 2015).

Application areas

The sort of applications in cisgenesis is limited by definition to native genes or alleles not yet present in the targeted elite plant material. Examples published comprise stacking R genes, for instance against late blight caused by *Phytophthora infestans* in potato (Haesaert *et al.* 2015; Haverkort *et al.* 2016) or against scab caused by *Venturia inaequalis* in apple (Krens *et al.* 2015). Possible applications in intragenesis may vary widely, including disease resistance, e.g. against *Botrytis* through combining the coding sequence of the strawberry polygalacturonase-inhibiting gene with the fruit-specific promoter from the strawberry expansin 2 gene (Krens *et al.* 2012), and drought tolerance through recombining vacuolar pyrophosphatase 1 with an endogenous drought-inducible promoter from dehydrin (Templeton *et al.* 2008, at that time still rated as "cisgenic"). Other examples are gene silencing, e.g. for improving product quality/composition, through RNAi (see section 4.6) and "rewiring" a developmental pathway, e.g. changing secondary cell wall formation to reduce lignin content (see under Synthetic biology section 4.8).

Barriers and drivers

The driver for cisgenesis was developing concepts that may increase consumer acceptance and simplify regulatory oversight as the types of genes used are basically the same as the ones already introgressed through traditional breeding methods (see under horizon). In addition, the advantage in comparison to classical introgression is the increased speed with which R genes can be stacked so as to diminish the possibilities for the pathogen to overcome the plant's resistance. This is particularly useful for crop species with long generation times and/or complex breeding due to heterozygosity, such as fruit trees and potato. It also enables maintaining the genetic make-up of successful varieties while adding the cisgenic traits, which is not possible by classical breeding as crops such as potato and apple are highly outcrossing and alternative systems of hybrid breeding have not yet been (fully) developed. Developments in sequencing and bioinformatics lead to an increase in knowledge on gene functions and effects of natural (allelic) variation, which could be used in both cis- and intragenesis. A complicating factor lies in achieving marker-free transformants, which is a less efficient system. The definition of cisgenesis poses a limitation on the types of traits attainable.

Horizon

For cisgenesis, a proof of concept has been delivered for late blight-resistant potato, including a resistance management system, using the cultivar Desiree in the so-called DuRPh project in the Netherlands (Haverkort *et al.* 2016). In Belgium, VIB (Vlaams Instituut voor Biotechnologie) is working on a version using the cultivar Bintje (VIB 2014), which is intended to be ready in 2018. Simplot's variant of the Innate potato containing a single R gene against late blight was deregulated in the US in 2015 (https://www.regulations.gov/#!docketDetail;D=APHIS-2014-0076). Interviews indicated that cisgenesis is seen as a typical example of a technical concept developed in an attempt to simplify regulatory concepts, though useful applications are worth considering in their own right. At the same time, commercial applications are thus dependent on regulatory interpretations and consumer acceptance, including labelling issues.

An intragenic application using gene silencing is already cultivated in the US, Simplot's Innate potato (see section 4.6).

4.6. RNAi – post-transcriptional gene silencing (PTGS)

Technical description

The technique uses an existing mechanism of post-transcriptional gene silencing (PTGS) to reduce gene expression in a targeted fashion. This is achieved by transformation with a construct containing an inverted repeat specific to the gene targeted. RNA transcribed from the construct forms double stranded RNA (dsRNA) by folding upon itself leading to a "hairpin". The hairpin is processed into microRNAs (miRNA), 20-21 nucleotides (nt) long, by a Dicer protein, DCL1, which is specific to the PTGS pathway. The miRNA, incorporated into a specialized Argonaute protein, AGO1, subsequently directs mRNA degradation or its translational repression leading to specific silencing of gene expression. The transgenic construct remains present in the final plant product in order to maintain gene silencing (Kamthan *et al.* 2015).

A special application of the method is using transgenic constructs targeted against genes of pests (e.g. insects) or pathogens (e.g. viruses or fungi), called "cross-kingdom" RNAi or host-induced gene silencing (HIGS) (Han & Luan 2015). In this case, dsRNAs are produced that, upon being taken up by the targeted pest or pathogen, are processed into miRNAs that interfere with the expression of essential genes (e.g. cytochrome p450 monoxygenase, vacuolar ATPase) of that organism.

Host effects

The technique leads to targeted gene silencing; therefore, in principle, a wide variety of traits achievable by knock-down mutants can be envisaged. Kamthan *et al.* (2015) discuss applications in plant architecture, fruit quality and shelf life, pathogen and pest resistances, several abiotic stress tolerances (e.g drought, salt, temperature), and biofortification. Off-target effects of silencing other genes may be possible, but little has been published about this (Senthil-Kumar & Mysore 2011; Casacuberta *et al.* 2015). There may also be some overlap with TGS (transcriptional gene silencing, also see section 4.7). In some cases, the hairpin RNA could also be processed into 24 nt miRNA (lmiRNA) that enters the DNA methylation pathway through loading onto AGO4 (Matzke & Mosher 2014). For *Arabidopsis*, a NERD protein has been described that induces RdDM (RNA-directed DNA methylation) using components of the PTGS pathway, including 21 nt siRNAs (Pontier *et al.* 2012). With RdDM, there is also the possibility of DNA methylation spreading into sequences adjacent to the RNAi target sequence (Casacuberta *et al.* 2015).

With the "cross-kingdom" RNAi, the range of organisms (e.g. insect species) targeted can be limited by careful choosing the gene sequence at which the dsRNA is directed (Burand & Hunter 2013). Whether non-target organisms (NTOs) will be affected, will depend on the extent to which specific gene sequences can be selected, which in turn will also depend on genome knowledge of (related) species (e.g. insect groups) and thus on developments in genomics and bioinformatics.

Application areas

"Classical" examples of genetic modification through plant transformation likely employed some way of PTGS, such as the FlavrSavr long shelf life tomato and low amylose potatoes (Krieger *et al.* 2008; De Vetten *et al.* 2003). Recently, a potato with improved product quality, i.e. low in bruise spot and heat-induced acrylamide formation (Simplot's Innate potato), and an apple with reduced browning (Okanagan's Arctic apples), both based on the system described in this section, i.e. by introducing inverted repeats, were deregulated in the US (Ye *et al.* 2010; USDA-APHIS).

Examples of applying "cross-kingdom" RNAi can be found particularly for insects, but also for fungi (*Fusarium*, Cheng *et al.* 2015) and oomycetes (*Phytophthora*, Sanju *et al.* 2015; *Bremia*, Govindarajulu *et al.* 2015). siRNAs can be transported through the phloem, which opens up the possibility of using grafted plants, for example a transgenic rootstock promoting silencing of a target gene in the scion. Zhao & Song (2014) showed that siRNAs produced from a transgenic hairpin construct based on the virus PNRSV in a sweet cherry rootstock were transported to the scion, which was accompanied by enhanced resistance to the virus. An alternative method of delivery of RNAi could become using an attenuated virus containing the dsRNA construct (Burand & Hunter 2013).

Barriers and drivers

A basic problem for PTGS can be the efficiency of gene silencing, which will usually be not 100%; at the same time, this could be advantageous for traits where diminished expression rather than complete knocking-out as in mutants is desirable. The effectiveness of "cross-kingdom" RNAi will also depend on the efficiency of uptake by the pest or pathogen, the specifics of the RNAi pathways in the organism, and the lethality of the process disturbed. For instance, RNAi appears to work not very well in flies (Diptera), which may be related to differences in the Dicer protein or the lack of other factors (Sid-1, Shreve *et al.* 2013). A recent improvement in effectiveness was achieved by expression in plastids (Colorado beetle: Zhang *et al.* 2015, *Helicoverpa armigera*: Jin *et al.* 2015). The advantage of expression in the chloroplast is that the dsRNA is not already processed into siRNAs in the plant host and so is delivered more effectively to the targeted insect. Transformation of chloroplasts has also been proposed as a way to mitigate pollen-mediated gene flow as plastids are usually transmitted through the mother line in Angiosperms (but this is not complete and varies somewhat between species, Stewart & Prakash 1998). With regards to applications in other insects, RNAi may not be able to compete with effective alternatives already offered by Bt (*Bacillus thuringiensis* toxin, a bacterial crystalline (Cry) protein introduced using "classical" plant transformation) against lepidopterans (moths). However, it may be attractive for control of groups for which no effective Bt is available, such as sucking insects, e.g. phloem-feeding aphids (Burand & Hunter 2013), though Bt adapted to such groups are being developed (Chougule *et al.* 2013). RNAi may also be helpful for pest insects such as Western corn rootworm that has relatively quickly developed resistance to Bt (Baum & Roberts 2014; Lombardo *et al.* 2016). Finally, there is an alternative delivery method for RNAi, i.e. applying it directly by topical application (as spray) (e.g. against virus, Robinson *et al.* 2014) and it will depend on relative efficiencies which method will be most competitive (see further Modifying gene expression with exogenous compounds, section 4.9).

With the fast developments in bioinformatics and DNA sequencing, knowledge is generated that can be used to improve the effectiveness and the specificity of "cross kingdom" RNAi. Particularly, recent work on (long) non-coding RNAs has contributed to the knowledge on the role of ncRNA in various pathways of gene regulation (Ariel *et al.* 2015; Liu *et al.* 2015) (see further section 4.7).

Horizon

Simplot's intragenic RNAi-based Innate potato is cultivated in the US (160 ha in 2015) and has just received regulatory approval in Canada. Likewise, Okanagan's Arctic apples have been deregulated in the US. BASF's Amflora "antisense" transgenic construct-based low amylose potato had been authorised in the EU for cultivation but was withdrawn shortly before commercialization (BASF 2012). Afterwards, the original EC's decision to authorise Amflora was annulled by the general Court of the EU (Case T-240-10, http://curia.europa.eu/jcms/upload/docs/application/pdf/2013-12/cp130160en.pdf). A Monsanto version of "cross-kingdom RNAi" against Western corn rootworm, combined with Bt against the same organism, has been recently authorised for cultivation in the US and Canada (Lombardo *et al.* 2015). Interviews indicated that the regulatory situation in the EU may as of yet not be favourable to investing in this type of work as these all are "classical" GM plants, i.e. containing transgenes. Thus, using topical applications (sprays) could be an interesting alternative (but see section 4.9).

4.7. RNAi – transcriptional gene silencing (TGS)

Technical description

The technique uses an existing mechanism of transcriptional gene silencing (TGS), RNA dependent DNA methylation (RdDM), to reduce gene expression in a heritable fashion. This can be achieved by transformation with a construct containing an inverted repeat specific to the gene (promoter) targeted. RNA transcribed from the construct forms double stranded RNA (dsRNA) by folding upon itself leading to "hairpins". This dsRNA is processed into small interfering RNAs (siRNA), 24 nucleotides (nt) long, by a Dicer protein specific to the RdDM pathway, DCL3. The siRNA, incorporated into a specialized Argonaute protein, AGO4, subsequently directs DNA methylation of the targeted promoter sequence leading to silencing of gene expression. Uniquely for higher plants, special DNA polymerases are involved in the RdDM pathway, Pol IV and Pol V, which are related to Pol II that is responsible for mRNA production (Matzke & Mosher 2014; Ariel *et al.* 2015).

The transgenic construct may be removed from the final plant product, e.g. by crossing. An alternative tool is using a virus vector to introduce the siRNA (Kasai & Kanazawa 2013). When the virus used is not seed-transmissible, the final plant product will remain free of introduced DNA sequences.

Host effects

The technique leads to targeted gene silencing; therefore, in principle, a wide variety of traits achievable by knock-down epimutants could be envisaged. As in PTGS (see section 4.6), off-target effects may be possible, i.e. silencing of genes showing homology in their promoters to the siRNAs produced by the constructs used. In addition, in absence of the transgenic RNAi-construct, DNA methylation is expected to be lost after some generations of multiplication. In the RNAi – PTGS section 4.6, already overlaps with the TGS pathway were discussed. Kasai & Kanazawa (2013) describe a special case with the use of a virus vector where TGS involving siRNAs is accompanied by suppression of the PTGS mRNA degradation pathway. If the transgenic construct is permanently present in the final plant product (as in RNAi – PTGS section 4.6) the effect is expected to be more stable.

Application areas

Among the examples mentioned by Kasai & Kanazawa (2013) are reduced flower pigmentation, male sterility and reduced amylose content.

siRNAs can also be subject to long distance transport through phloem, which opens up the possibility of using grafted plants, for example a transgenic scion promoting silencing of a target gene in the rootstock. Regenerating plants from such a rootstock could lead to an epimutant completely free of exogenous DNA sequences (Kasai & Harada 2015). Gene silencing through long distance transmission was even achieved by agro-infiltration in the scions (Kasai & Kanazawa 2013).

There are also reports on mutants of the *MSH1* gene showing large changes in DNA methylation patterns that are heritable, transmissible between graft partners and that lead to increased growth vigour in *Arabidopsis* (Virdi *et al.* 2015) and tomato (Yang *et al.* 2015). It is not yet clear to what extent this will lead to applications in breeding.

An example of using the technique without removing the transgenic construct is a hybrid seed production system in maize using the *Ms45* nuclear male fertility gene, which in a homozygous recessive (silenced) state leads to male sterility. The male-sterile female parental line is created by crossing two complementary male fertile lines, each containing the (fertility) *Ms45* allele, but with different promoters and different RNAi silencing constructs as follows: one line combining *Ms45* with a heterologous (active) promoter and an RNAi construct silencing the heterologous promoter of the other line and vice versa. Thus, the progeny of this cross contains two *Ms45* alleles, the promoters of which are each silenced by the inverted repeat carried by the other allele, leading to male-sterile plants that can be used as female parent in hybrid seed production (Cigan *et al.* 2014).

Barriers and drivers

A basic problem for TGS is the efficiency of gene silencing, which may vary to an as yet unknown extent among genes, and the stability of the TGS in the absence of the inducing construct. DNA methylation is maintained by factors such as methyltransferases, e.g. MET1, but can also be removed by factors such as DNA glycosylases, e.g. ROS1. Kasai & Kanazawa (2013) showed a higher effectiveness of using a virus vector, cucumber mosaic virus (CMV), as vector for RNAi constructs. Disadvantages of such an approach are the limitation to particular hosts by the specificity of particular viruses and to seed-propagated plants.

With the recent developments in bioinformatics and sequencing, in particular transcriptomics (RNA sequencing), knowledge on non-coding RNAs (ncRNA) is expanding quickly (Liu *et al.* 2015), including the development of databases for ncRNAs (Xuan *et al.* 2015, Patra *et al.* 2014). This may become a driver of RNAi-based methods, as there is still much unknown about mechanisms of RNA-based signalling pathways and control of development in plants, in which also histone modifications play a role (Matzke & Mosher 2014). At the same time, examples in phosphate homeostasis (Liu *et al.* 2015) and flowering control (vernalization, Chekanova 2015) indicate a high complexity of regulation, which may hamper relatively simple applications such as those targeting single genes. In addition, alternative pathways of RNAi TGS have been described for Arabidopsis (Bond & Baulcombe 2015). Genome editing techniques, in particular CRISPR-Cas (see section 4.2), have begun to be used for testing functionality of ncRNAs (Basak & Nithin 2015).

Horizon

Interviews indicated that product stability is adamant to breeders' commercialization decisions (cf. the S for stability in the DUS prerequisites for plant variety registration) and this is not clear for RNAi systems using DNA methylation in which the transgenic construct has been removed. In addition, there is the uncertainty around the status of "null segregants" (see section 4.4) with regard to EU GM regulation. By maintaining the transgenic transcriptional silencing construct as in RNAi – PTGS (section 4.6), more stable applications are possible, such as a female inbred maintaining system for hybrid seed production, but this has the regulatory disadvantage for classical transgenesis already mentioned in the same section 4.6.

4.8. Synthetic biology

Technical description

"Synthetic biology" does not appear to be a well-defined field, as different authors use it for different types of approaches. For instance, genome editing techniques, such as CRISPR-Cas, sometimes are included, but in our classification we reserved this term for various alternative, novel uses of the CRISPR-Cas protein, such as changing it into genome site-specific activators/repressors of gene transcription. Definitions of the term entail engineering plants for new functions or devices (e.g. sensors) (Liu and Stewart 2015) that are not found in nature, in other words, intentional design of artificial biological systems (Lusser *et al.* 2011). The field is still in its infancy and therefore we will limit ourselves to a few examples. The furthest developed branch appears to be the introduction of novel (metabolic) pathways. Further away, there is the construction of synthetic chromosomes still in the research phase (Birchler 2015) that will be briefly mentioned here.

Host effects

Introducing novel pathways means changes in (relative) amounts of metabolites or proteins or the introduction of products totally new to the plant, such as pharmaceuticals. Effects will depend on the kind of pathway introduced and the way of regulating its expression.

Novel pathways could be systematically introduced through synthetic chromosomes. Minichromosomes have been engineered by truncating a B chromosome in maize and adding a transgenic array capped with a telomere at one end to the remaining centromere of the B chromosome. For enabling the later addition of novel sequences, a sequence for site-specific recombination could be inserted (Birchler 2015).

The CRISPR-Cas system that is presently widely used for genome editing (see section 4.2), originally functions in controlling viruses (bacteriophages) in archaea and bacteria. Ali *et al.* (2015) reported that CRISPR-Cas9 can also be targeted in that manner in plants by overexpressing Cas9 and providing guiding RNA (sgRNAs) specific to virus, in this case TYLCV. Virus DNA accumulation was shown to be significantly reduced; the system could be extended to other viruses by introducing adapted sgRNAs.

The specific DNA sequence recognition function of the Cas protein may also be used for other purposes than genome editing. By knocking out the DNA nuclease function (dCas9) and combining with alternative active domains affecting transcription, the Cas/guide RNA complex can be used as artificial transcription factor to enhance or decrease gene expression, or to make it inducible upon demand, e.g. in response to the addition of a particular chemical compound. Finally, a complex can be engineered to modify DNA epigenetically (e.g. by adding DNA methylating activity, see RNAi-TGS section 4.7, to the dCas9) in order to alter gene expression genome-wide (Puchta 2016; Thakore *et al.* 2016). Very recently, Komor *et al.* (2016) added another variant, i.e. combining dCas with a cytidine deaminase enabling to change a single cytidine to uridine (thereby effecting a C to T substitution) without making a dsDNA break (in effect a specialized form of genome editing, see section 4.2).

Application areas

The classic example of adding a pathway for biofortification is the Golden Rice with increased levels of pro-vitamin A, which in its improved version has a transgenic construct of a phytoene synthase from daffodil combined with the originally used carotene desaturase from *Erwinia uredovora* (Paine *et al.* 2005). More extensive adaptation of pathways for biofortification has been described for maize by Zhu *et al.* (2008) and Naqvi *et al.* (2009; 2011). They used combinatorial transformation to enhance vitamin production in endosperm, which meant introducing 5 transgenic constructs, including various endosperm-specific promoters, simultaneously through a biolistics approach followed by selecting plants expressing several or the complete set of transgenes for their production efficiency. In this way, Zhu *et al.* (2008) increased vitamin A (carotenoid) production and Naqvi *et al.* (2009) increased production of even three vitamins at the same time: β-carotene, ascorbate and folate.

An example that could count as a special application of intragenesis (see section 4.5) is the "rewiring" of a developmental pathway, e.g. changing secondary cell wall formation to reduce lignin content and increase polysaccharides for biofuel production (Yang *et al.* 2013). Lignin deposition was directed to

vessels by placing the cinnamoyl CoA 4-ligase (*C4L*) gene under the pVND6 promoter in an *Arabidopsis c4l* mutant in order to keep vessel formation (and thus water transport) effective while fibres remained low in lignin and at the same time had a higher cell wall polysaccharide deposition by the introduction of an artificial positive feedback loop using the master fibre transcription factor NST1.

In the border area between "white" and "green" biotechnology, already for some time the possibilities of using plant (cells) for the production of high value compounds, in particular pharmaceuticals, is being studied. For instance, monoclonal antibodies can be produced in plants. In addition, antigens for use as vaccines can also be produced in plants and thus, could also be applied orally (recent review: Juarez *et al.* 2016).

Barriers and drivers

For pharmaceutical purposes, technical hurdles had to be overcome, such as adjusting protein glycosylation patterns to mammalian (human) patterns. Even though there may be clear advantages to using plants for pharmaceutical production (cost effectiveness, safety as they will be largely free of human pathogens, often more simple purification), it appears to be difficult to compete with existing production systems (Juarez *et al.* 2016). Moreover, the potential admixture of therapeutic production systems with food production in the same crop, particularly in plant species with seeds attractive for pharmaceutical production purposes, such as maize, will call for tight containment schemes (e.g. Mascia & Flavell 2004). Alternative uses of CRISPR-Cas and synthetic chromosomes still appear to be in the research phase.

Horizon

Of metabolic pathways for biofortification, Golden Rice is still in the phase of multi-location field trials for optimizing yields (IRRI 2014). Up to now, the only therapeutic that has been approved (by the FDA in the US) is a human enzyme, taliglucerase α, for the rare lysosomal storage disorder, Gaucher disease, produced in carrot cells by the Israelian company Protalix. The interviews indicated that, partly depending of what one counts as synthetic biology, most of it is too far away from practical applications and/or showing unclarities about the position in safety assessments/regulation to be fit for applications. Engineering metabolic pathways and/or production of pharmaceuticals could be interesting, provided that the production can be strictly contained, particularly with regard to crops species also used in food production. Thus, measures to avoid gene flow or any other admixture between a pharmaceutical line and the conspecific food crop need to be in place. Other interesting applications are in the field of bio-economy, e.g. low lignin (see the "rewiring" *Arabidopsis* biofuel example above under "Application areas", also "classical" transgenic examples in poplar have been tested in the field, cf. Van Acker *et al.* 2014).

4.9. Modifying gene expression with exogenous compounds

This is a category outside of the central theme of this study, i.e. modifying gene expression in a heritable fashion, but because of overlap with developments described above it will briefly be touched upon here.

Implementing RNAi against pathogens and pests (see RNAi-PTGS section 4.6) may take the form of topical application of dsRNA solutions (e.g. against virus, Robinson *et al.* 2014; against insects, Baum & Roberts 2014). Their effectiveness depends on the development of formulations with delivery agents that improve delivery to the pathogen or pests and environmental stability (Baum & Roberts 2014). Efficient delivery solutions may also be used to direct gene expression in plants, for instance to improve shelf life or to induce drought tolerance during a period of water deficit (for review, Hu & Lübberstedt 2015).

A special variant of such crop management by spraying is by engineering ABA receptors to be responsive to specific agrochemicals, so that ABA signalling can be activated by spraying an agrochemical during drought spells (Rodriguez & Lozano-Juste 2015).

Agro-infiltration, including use of virus vectors, is still mainly used for research purposes. It can also be used for high value protein production (see Synthetic biology, section 4.8). Special variants, early flowering induced by using a virus vector and TGS in rootstocks by agro-infiltration in the scion, are discussed above (Plant transformation with transgenes not in end products section 4.4 and RNAi-TGS section 4.7, respectively).

"DNA-free" genome editing through SSN delivery as protein or transient expression in cells is discussed above under Genome editing section 4.2.

Interviews indicated that for many breeders sprays are outside of their business models, but it could be interesting for growers to increase flexibility and yields, and to simplify the choice for a particular variety to grow, which normally often involves uncertainties about expected water conditions and pest/pathogen levels. Regulatory aspects have been discussed above under RNAi – PTGS section 4.6.

4.10. Regenerating a species hybrid from a graft junction

In 2009, Stegemann & Bock reported that they were able to regenerate shoots from cells in tobacco graft junctions in which the cytoplasmic (plastid) genomes and nuclear genomes of scion and rootstock were exchanged. They used different transgenic antibiotic resistances and fluorescent reporter genes to select for and confirm these new combinations of plant genetic materials. These new combinations are like plant products that can be obtained by cell fusion (somatic hybridization), which is an established technique mainly used for introducing CMS (cytoplasmic male sterility) in crop plants (Van de Wiel *et al.* 2010). However, in this case it is not clear by what mechanism cytoplasmic and nuclear genomes become exchanged. In a next step, Fuentes *et al.* (2014) showed that they could also regenerate a new allopolyploid species hybrid from a graft junction between *Nicotiana tabacum* and *N. glauca*. Here the new combinations of the two nuclear genomes were again selected by using different transgenic antibiotic resistance and reporter genes. Apart from the use of transgenic selection markers, the techniques used here appear to be in the realm of conventional breeding: grafting and plant regeneration through tissue culture. Nevertheless, these intriguing observations may open up a novel way to generate difficult species hybrids. As these are as of yet single research examples, it is not yet clear to what extent this method may lead to commercial applications in breeding.

5. Discussion and conclusions

The aim of this study was to provide an overview of new developments in plant biotechnology. These encompass both novel techniques such as genome editing and new concepts or applications for already established techniques such as plant transformation (transgenic plants). We performed a literature review and held interviews with stakeholders. The interviews followed the list of techniques presented above (cf. Table 1 and Annex) and their results were mentioned with each technique discussed, mostly in the Horizon paragraphs. The interviews were especially helpful in identifying drivers and barriers (particularly those perceived with regard to regulatory uncertainty) and the (short term) horizon of products of the techniques. They generally confirmed that we had not missed relevant developments. Nevertheless, one needs to take into account that companies basically may not be able to freely discuss every novelty as they may feel the commercial need of protecting their business strategy.

We will first discuss the classification of techniques that we followed here. Subsequently, we will discuss intended and unintended effects of the techniques, followed by drivers and barriers for their application, and finally the horizon, *i.e.* what kind of applications can be expected in the short and medium term.

Classification

We presented our overview of developments in plant biotechnology by a listing of techniques and/or concepts as follows (see Table 1): genome editing, alternative uses of classical transformation technology, RNAi (specific gene silencing) methods and synthetic biology, plus remaining categories of external compounds directing gene expression and an additional development in grafted plants. There are, however, many cross-connections among techniques and concepts. Thus, not only "Plant transformation with transgenes not in end product" but actually the greater part of these techniques have as common denominator a step involving transgenic lines, with the transgene not being part of the final plant product. Only the genome editing variant SSN - HR (with foreign sequences), cisgenesis/intragenesis and RNAi-PTGS (and some applications of RNAi-TGS) rely on the presence of the introduced sequence or transgene in the final plant product. In an attempt to visualize cross-connections, an alternative scheme shown in Table 2 presents the developments according to the way transgenic constructs are used: plants with transgene insertion(s), with transgene insertion followed by removal, and transient expression/presence or even not using any (transgenic) DNA but (ribo)proteins of RNA for changing traits. This scheme further shows that there are multiple ways of implementing genome editing technology, including alternative forms of CRISPR-Cas, and of using RNAi, and the use of virus vectors as alternative to "classical" transformation. "Classical" use of transformation (with the transgene(s) in the final plant product) also shows further developments with novel traits, such as introduction of metabolic pathways involving larger numbers of (recombined) genes, but these may not be immediately obvious as category, as we classified this more or less arbitrarily with Synthetic biology (apart from the special cases of cis/intragenesis). Classification could also be based on the nature of the final plant products, such as plants with new genes at a new chromosomal locus, plants without new genes but with a mutation and plants without new genes or new modifications, which was used in Schaart et al. (2016).

A special case is the use of grafted plants. We included the use of a transgenic rootstock under "Plant transformation with transgenes not in end product", whereas for instance, Lusser *et al.* (2012) had a separate category for it. Grafting is indeed special in that part of the plant cultivated contains the transgene, in the particular example the rootstock, and so the harvests from the scion, do not. On the other hand, apart from providing resistance to soil-borne diseases, such grafted plants can also be used in both forms of RNAi (PTGS and TGS) by providing transportable siRNA that can influence gene expression in the scion, up to a variant where a TGS plant without transgene is regenerated from a rootstock silenced by RNA from a transgenic or infiltrated scion. Such links between uses of the technologies are also referred to as much as possible in the individual sections of chapter 4.

Intended and unintended effects

As in classical plant transformation, intended and unintended effects will largely depend on the type of novel trait introduced and so will vary from case to case. Genome editing and RNAi techniques, and potentially synthetic biology, enable changing a wide variety of traits, e.g. abiotic stress tolerances, disease resistances or metabolic changes underlying product quality. With genome editing (except for SSN – HR using "foreign" sequences as template) and RNAi, existing ("native") plant genes are changed. ODM can produce gene knock-outs and small changes of one amino acid. SSN – NHEJ will mainly produce gene knock-outs. Thus, with SSN – NHEJ mainly recessively inherited traits will be produced, while with ODM additionally (co)dominantly inherited traits may be possible, that is, those based on single amino acid changes. SSN – HR enables producing precise changes, from small sequence changes up to inserting complete (novel) gene sequences, and thus dominantly inherited traits (allelic variants or novel genes). With RNAi, gene knock-downs are produced, but as opposed to genome editing, they will inherit dominantly, as long as the silencing construct is present in the plant line. With "null segregants", such as in early flowering, a large array of traits can be introduced, but in this case, apart from using the transgenic line for improving efficiency, conventional crossing methods are used, and so both recessively and dominantly inherited traits may be produced based on existing genes in the cross-compatible germplasm.

Interesting examples of effects directly related to a specific trait are found in resistances to insects or pathogens. One of the recent trends is knocking out/silencing so-called S (susceptibility) genes (Pavan *et al*. 2010). This could lead to unintended effects, i.e. changes in plant performance and/or affecting non-target organisms (NTOs). An intriguing example of avoiding the first is using genome editing for making a small change in the promoter of an S gene in rice abolishing the target of a bacterial pathogen without affecting the promoter regions used for the gene's expression necessary for plant performance (Li *et al.* 2012).

Resistance to insects or pathogens through "cross-kingdom" RNAi or host-induced gene silencing (HIGS, a special application of PTGS) is another interesting trend and may represent a case of a truly "novel" type of trait, though exchange of small RNAs between organisms is an existing phenomenon (cf. Han & Luan 2015). This trait is based on a transgenic construct maintained in the final plant product. The transgenic construct introduced encodes a dsRNA targeted at vital functions of the pest or pathogen. By carefully choosing the gene sequences targeted in the pest insect, effects on non-target organisms (NTOs) could be minimized to an even larger extent than with Bt. The occurrence and magnitude of side-effects will depend on the possibilities of identifying suitable target sequences that are specific for the target organism only. It can be expected that increased availability of genome sequences will increase the ability to find such sequences, and if genome sequences are not available yet, it has become cheaper and easier to produce them for the purpose of such a project.

As the greater part of these techniques uses the transgene during the process but not in the final (plant) product, unintended effects from the transgenes are lacking, and any effect from the transformation process on the genome of the transgenic line will be minimal as most of its genome will be removed by repeated backcrossings during the breeding process towards varieties. For SSN-based genome editing techniques there is even an alternative to avoid transformation altogether, employing transient expression of the construct encoding the SSN in the cell and regenerating plants lacking transgenic insertions in their genome. A step further in this direction is introducing the proteins and/or gRNAs directly into protoplasts (DNA-free genome editing). For the "null segregant" approaches, such concepts are also explored, for instance in early flowering. An interesting variant published for early flowering is introducing expression constructs through virus vectors, which are subsequently removed, e.g. in case the virus is not seed-transmissible, so will not end up in the progeny. Examples of using virus vectors are also reported for genome editing and RNAi (see Table 2). Notably, for many of the "null segregant" approaches, removing the transgene is inherent to effective usage of the technique, so not primarily aimed at any unintended side effects. For example, the transgenic early flowering trait is useful during the (back)crossing process but is unwanted in the end product.

HR (homologous recombination) application of SSNs could provide a more precise way of introducing desirable alleles, e.g. by exchanging them with alleles present in the elite material. In this way, (cis)genes could also be introduced at precise locations in the genome in order to reduce unintended effects to the minimum. Further development of NGS and bioinformatics will provide clues to desirable

alleles or cisgenes by allele mining/modelling, for instance in wild/exotic plant accessions. HR is however still technically difficult and having off-target effects for which solutions are being researched.

Depending on the specific variant, off-target mutations (unintended modifications at other chromosomal locations) are possible with genome editing. For CRISPR-Cas9, in the highly active field of medical-biological research, improvements have been made to the Cas9 protein or gRNA in order to reduce the off-target effects. In addition, such effects could now be identified (to some extent) using NGS and bioinformatics (genome re-sequencing) (see e.g. Kim *et al.* 2015). One needs to place this in the context that genome editing is expected to be far more precise than classical mutagenesis (which also induces multiple random mutations that are not related to the trait of interest), and that plant breeding involves extensive phenotypic and increasingly genotypic testing for selecting elite materials for commercialization (for instance, removing unintended phenotypes from materials generated by classical mutagenesis, cf. EFSA GMO Panel 2012a).

Likewise with RNAi, though aimed at specific gene sequences, off-target silencing also appears to be possible, but relatively little has been reported about it. NGS and bioinformatics (transcriptome sequencing) could be helpful in assessments here as well. It might be complex however to take into account all potential variability in a crop species, as small sequence changes already will have effects with the short sequences (21-24 nt) used in RNAi. An alternative approach could be to compare intended and unintended target mRNAs from the plant with the RNAi construct and an isogenic line in sequence libraries of RNAi-cleaved mRNA made using PARE (parallel analysis of RNA ends) (Casacuberta *et al.* 2015). The recently increased attention to ncRNAs in fundamental research may be helpful (e.g. Liu *et al.* 2015), also in gaining more knowledge on RNAi mechanisms and the various pathways leading to PTGS and/or TGS.

Drivers and barriers

An important general driver for many of the new developments is the search for concepts that may simplify compliance with regulatory demands or that may be outside of regulatory oversight. At the same time, as long as the status of techniques with regard to regulatory oversight in the EU is not clear, they are not likely to be applied directly for producing marketable products. Cisgenesis is a case in point: the use of genes in their native state from the crop and related species themselves was expected to simplify regulatory assessment of the plant products and to improve consumer acceptance. Subsequently, a proof of concept was delivered, e.g. in late blight resistant potato (Haverkort *et al*. 2016), but commercial application has not yet taken off in view of the unclear regulatory status. As long as the legal situation is regarded as complex or costly, it may even be the case that breeders use all available novel technology plus "big data" (sequencing technology and bioinformatics) in their research (leads, traits), but for commercial application subsequently "reconstruct" the promising plants using conventional means, such as mutagenesis. For example, one could test mutations in various genes using CRISPR-Cas9, identify a mutation in a gene that has the desired phenotypic effect, then search such a mutation in a population generated by mutagenesis using powerful new DNA sequencing methods for efficient screening. The mutated allele is subsequently used in the normal breeding program.

The large progress in sequencing (NGS, RNA seq) and bioinformatics has already been mentioned and is generally an important background driver for many of the new techniques. Data produced using these techniques may provide clues for genes to be targeted by the genome editing techniques, while in turn these techniques, particularly CRISPR-Cas9, can serve as a powerful tool for functional genomics, i.e. to test for the functions of genes/alleles or ncRNAs (Basak & Nithin 2015). NGS and bioinformatics also provide wider knowledge on RNAi pathways, possibly also for methods such as TGS (RdDM) for which the expected stability of the plant product (i.e. the DNA methylation) hampers commercial application in the case that the transgenic construct is removed from the final plant product. In addition, an upcoming field is the finding of the common occurrence of long non-coding RNAs in the genome, including various examples of how they could be involved in regulating gene expression. For instance, various lncRNAs transcribed from the flowering gene *FLC* in *Arabidopsis* were shown to be involved in regulating vernalization (Chekanova 2015). This field is in its infancy and practical applications have not yet been identified.

Horizon

Table 3 presents an overview of the techniques with the horizon of applications. In some areas, developments are going fast, particularly in genome editing. For the ODM variant, the HT oilseed rape (SU canola) developed by Cibus has been brought to the market in the US and was recently authorised in Canada. In the SSNs, variants are quickly following upon one another, with the most striking development being CRISPR-Cas9. This technique was not even mentioned in the review of novel plant breeding techniques by Lusser *et al.* in 2012, but is now most promising due to its relatively easy applicability for a wide array of possible traits. The main focus of the genome editing techniques is introducing targeted changes in the genome, which is an improvement compared to random changes in classical mutagenesis. This even enables inducing changes that would be unlikely to be achieved by traditional mutagenesis (see the example of an S gene in rice above under "Intended and unintended effects").

Another interesting development is the use of "null segregants", i.e. the use of transgenic plants at some stage in breeding or seed production, but the final product does not contain the transgene. These are used for speeding up the breeding process, e.g. in early flowering in crop species with long generation times such as fruit trees, or for implementing a particular step in the process, such as haploid inducers. An example of early flowering is the FasTracking system in plum. An example in hybrid seed production, Pioneer's SPT system using a transgenic maintainer line, is already applied in the US.

RNAi applications range from a product quality example in potato already cultivated in the US (Simplot's Innate potato) to interesting developments in disease and pest resistance for which one example against insects was recently authorised in the US (against Western corn rootworm in maize). TGS in the form of RdDM (with removal of the transgenic construct) is unlikely to be effective in the short term for lack of trait stability.

Whether synthetic biology is far away from applications partly depends on how it is defined. Engineering metabolic pathways is already developing for some time following various concepts: introducing a series of transgenes encoding several vitamin pathways for biofortification, or even rewiring pathways by rearranging genes and promoters within the intragenesis concept. A high-value example is the production of pharmaceuticals for which plants have advantages as providing clean products relatively cheaply (e.g. free of human pathogens). Glycosylation patterns have been adapted to mammalian/human requirements through extensive research. There is only one product authorised at present, against an orphan disease (Gaucher). Finally, engineered Cas9 without nuclease activity (dCas9) can be used to build novel transcription factors or epigenetic modifiers (Puchta 2016). Whether this remains a research tool or will be used in transgenic commercial applications, remains to be seen.

Generally, the products of the techniques presented in this report need to cross the usual "valley of death" for innovations to achieve a commercially viable application. This can range from technical or practical barriers with the specific crop or trait to competition with all sorts of alternative approaches. Concrete examples have been discussed for engineered metabolic pathways e.g. the biofortified Golden Rice still being optimized for agronomical performance and pharmaceuticals in plants. Apparently, pharmaceutical plant production systems still can hardly compete with established animal or microbial cell systems and there is the added need for careful segregation in the case of using food crops (Juarez et al. 2016). As another example, RNAi can be developed for taxonomically diverse groups, but in groups where there is a sustainable Bt alternative, RNAi may not be competitive with regard to effectiveness, although it could be useful in combination with Bt to mitigate resistance development in the pest insect. There may be limitations in getting the dsRNA at the right place in the insect and here an alternative may be to use sprays (Baum & Roberts 2014). For this reason, sprays affecting gene expression are briefly described in this report. Sprays are further outside the scope when they are not changing genomes, and actually belong to the field of pesticides. Furthermore, silencing using RNAi will often not be absolute, which may hamper the application for certain purposes, but may be desirable for other traits where complete knocking out has undesirable side effects.

Conclusions

Predictions on developments in green biotechnology need to be considered with care as shown by the recent overwhelming success of CRISPR-Cas, which was unknown as a genome editing tool in plants just five years ago. As already seen in classical GM (transgenic plants), highly promising applications need to go through the often painstaking process of achieving commercial viability, i.e. making it into elite varieties that are competitive or superior to existing varieties. A practical problem with predicting the likelihood of development into commercial products is the additional focus on their regulation. GM regulation and labelling are perceived as prohibitively costly and the regulatory process as extremely uncertain (in the EU). These regulatory aspects were connected to ideas about consumer acceptance; both were not the focus of our review but proved inescapable in discussions on drivers and barriers, as experienced in interviews. Table 3 presents an overview of the techniques with an attempt to depict the horizon of applications, disregarding the expected impact of regulation in the EU as far as possible.

The most likely techniques to show strong developments in the near future are the genome editing ones, in particular CRISPR-Cas9. The "null segregant" concepts are promising for accelerating the breeding process and for producing F1 hybrid seeds. Some RNAi applications are already on the market outside of the EU and others may become relevant in the near future, e.g. "cross-kingdom" RNAi, but others, e.g. TGS (RdDM), at least in their variant of the end product not containing the transgenic silencing construct, are still awaiting effective examples; possibly, a better insight into mechanisms and stability of epigenetic changes will be helpful with the latter. In the synthetic biology field, applications in metabolic engineering may become relevant soon, particularly in bio-fortification, bio-based economy applications and possibly, pharmaceuticals. As with "classical" plant transformation, effects, intended as well as unintended, will largely depend on the type of trait introduced. Many of the traits are in the same categories as in "classical" transformation and/or conventional breeding, such as product quality, metabolites, abiotic and biotic stress tolerances, but are expected to be achieved with more efficiency and/or precision. We discussed some examples of alternative ways for achieving resistance and/or more precision attainable in resistances against pathogens and pests, in terms of plant performance and specificity for the target organism.

Table 2. *Overview of different uses of transgenic constructs and technologies*

For stable DNA integration into the genome.	For stable DNA integration into the genome, but not in the end product.	For transient expression.
Delivery by *Agrobacterium*-mediated transformation or by direct DNA transfer (biolistic-, whisker-, PEG-, electroporation-mediated)	Delivery by *Agrobacterium*-mediated transformation or by direct DNA transfer (biolistic-, whisker-, PEG-, electroporation-mediated)	Delivery by *Agrobacterium*-mediated transformation or by direct DNA transfer (transient expression from plasmid), mRNA, protein or ribonucleoprotein transfer. Also virus-mediated (for overexpression and VIGS)
Intended for continuous presence of novel DNA sequences: -repair template DNA introduced by SSN-induced HR -cisgenesis (eg R-genes); intragenesis -RNAi-PTGS, e.g. against insects (cross-kingdom RNAi) -RNAi-TGS (maintained silencing) -CRISPR-Cas9 against DNA viruses -synthetic biology – metabolic pathways -rootstocks of grafted plants	Intended as intermediate step and transgene will be removed in later generations (e.g. by segregation): -in genome editing (NHEJ or HR) -to facilitate, accelerate breeding (induced early flowering, several elements of hybrid variety breeding and hybrid seed production) -rootstocks of grafted plants: no transgene in scion products	Intended for temporary presence of DNA, mRNA, protein or ribonucleoprotein (all these will be spontaneously degraded in the cell but DNA may also become stably integrated unintendedly and requires here check for absence): -mutation induction by ODM, SSN (NHEJ) -HR induction (SSN-HR) -RNAi-TGS (methylation pattern is maintained over generations) -RNAi-TGS (VIGS) -induced early flowering (virus) -signalling from rootstocks of grafted plants to scions and v.v.

Table 3. *Overview of techniques with their applications, including the horizon basically without regard to barriers perceived with regulatory uncertainty in the EU*.

Technique	**Applications**	**Barriers / Drivers**	**Horizon**
Genome editing: Oligo-directed mutagenesis (ODM)	Wide array (potentially): (1) Herbicide tolerance (HT) (2) Modified product quality; stress tolerances biotic (S genes) & abiotic (drought), etc.	Technically demanding: protoplast technology & selection efficiency Regulatory / Precise mutagenesis method without use of transgenic constructs	1 Short term: HT marketed outside EU 2 Medium term: other types of traits
Genome editing: Sequence-specific nucleases (SSNs): non-homologous end joining (NHEJ)	Wide array (gene knock-out): HT; modified product quality; stress tolerances biotic (S genes) & abiotic, etc.	Off-target effects Regulatory & IPR / Precise mutagenesis method, could also be used by transient expression or "DNA-free" CRISPR variant relatively easily applied Genomics for gene functionality & identifying off-target effects	1 Short term: outside EU, one product considered outside of regulation by USDA-APHIS 2 Medium term: CRISPR products
Genome editing: Sequence-specific nucleases (SSNs): homologous recombination (HR)	Wide array (incl. new genes/promoters & stacking): HT; modified product quality; stress tolerances biotic (R genes) & abiotic, etc. "Gene drive"	Technically demanding: low HR efficiency (also for "gene drive") Off-target effects Regulatory / Precise method of mutagenesis/gene replacement or insertion at specified location/gene stacking Genomics for gene functionality & identifying off-target effects	1. Short term: proof of concept but no product marketed 2. Medium term: possibly gene stacks; also allele replacement (latter still technically demanding)

Technique	Applications	Barriers / Drivers	Horizon
Plant transformation (new variants): Plant transformation with transgenes not in end product	• Induced early flowering for accelerated breeding in fruit trees (wide array of traits, e.g. biotic stress); • Suppression of meiotic recombination (reverse breeding), maintainer lines, or haploid inducers supporting hybrid breeding; • transgenic rootstocks (wide array of traits, e.g. soil-borne stresses)	Regulatory / Increased efficiency of breeding without transgenic constructs in end product; could also be used by transient expression (virus vector) in early flowering	1. Short term: hybrid maintainer system used outside of EU; early flowering & transgenic rootstocks proof of concept but no product marketed yet 2 Medium term: possibly haploid inducers
Plant transformation (new variants): Plant transformation introducing genes from cross-compatible species	• Cisgenesis: genes from cross-compatible species, mainly R genes • Intragenesis: novel combinations of genes & promoters from cross-compatible species: wide array of possibilities for changing gene expression levels and metabolic/developmental pathways	Cis limitation to “native” genes Regulatory / Cis use of “native” genes; intra wide possibilities (without introducing new genes)	1 Short term: cis one product deregulated outside EU, which is a stack with an intra RNAi variant already marketed outside of EU 2. Medium term: possibly other cis & intra products marketed
RNAi: Post-transcriptional gene silencing (PTGS)	Wide array (gene silencing): modified product quality; stress tolerances biotic & abiotic, etc. Special variant: tolerance biotic stress through “cross-kingdom RNA”	Silencing efficiency Off-target effects Alternative of topical application Regulatory (transgenic product) / Precise method of silencing gene expression, incl. “cross-kingdom”, with dominant inheritance & potentially more subtle regulation of expression levels (as opposed to gene knock-outs) Genomics for gene functionality & identifying off-target effects	1 Short term: intragenesis variants marketed outside of EU; a “cross-kingdom” RNAi variant deregulated in US and Canada 2 Medium term: possibly more examples of “cross-kingdom” RNAi

Technique	Applications	Barriers / Drivers	Horizon
RNAi: Transcriptional gene silencing (TGS) (RdDM)	Wide array (gene silencing): modified product quality; stress tolerances biotic & abiotic, male sterility for hybrid breeding etc.	Silencing efficiency & stability (with transgene removal) Off-target effects Regulatory (i.p. transgenic product variant) / Precise method of silencing gene expression & potentially more subtle regulation of expression levels (as opposed to gene knock-outs); also possibility of using virus vector not carried over through meiosis or possibility of regenerating epigenetically changed plant from non-GM part of grafted plant Genomics for gene functionality & identifying off-target effects & epigenetic stability	1 Short term: research phase, i.p. variant with transgene removal 2 Medium term: possibly variants maintaining transgene
Synthetic biology	• Introduction of novel pathways; "plant as factory" (pharmaceuticals/antibodies) • Site-specific activators/repressors (targeted activation or repression of gene expression by artificial factors affecting transcription or inducing epigenetic changes using combinations of active domains with SSN-derived DNA-binding domains) • Adapting CRISPR-Cas9 to targeting plant DNA viruses • Synthetic chromosomes	Competitiveness with microbial systems & segregation from food chain for pharmaceuticals Regulatory (transgenic product) / Possibility of producing valuable compounds or plants for pharma, and bio-economy & -fortification	1 Short term: one pharmaceutical authorised 2 Medium term: bio-fortification & possibly bio-economy variants; others in research phase, i.p. synthetic chromosomes

Technique	**Applications**	**Barriers / drivers**	**Horizon**

Modifying gene expression with exogenous compounds	• Gene silencing by topical application of dsRNA:
	  ○ to plant (parts);
	  ○ to insects/pathogens
	• Agro-inoculation, VIGS
	• “DNA-free” genome editing (by SSN delivery as (ribonucleo)protein, mRNA or transient expression of SSN-genes in cells (see above)
		Topical application could compete with transgenic RNAi versions above but likely in regulatory realm outside scope of present study
		Agro-inoculation mainly research tool
		“DNA-free” genome editing, see above
			For most breeding companies outside of business model & outside scope of present study

Other: regenerating species hybrid from graft junction	Species cybrid or hybrid (potentially wide array of traits)	Regulatory (transgenic selection markers) / Possibly interesting development	Two publications, in research phase
Not addressed	Transforming plant pest insects; algae		

6. Abbreviations

Bt *Bacillus thuringiensis* toxin

CCE centromere-mediated chromosome elimination

CRISPR-Cas clustered regularly interspaced short palindromic repeats – CRISPR-associated protein, a type of SSN

DSB double-strand break

dsRNA double-stranded RNA

GT gene targeting

HDR homology-driven repair

HIGS host-induced gene silencing

HR homologous recombination

HT herbicide tolerance

Indel insertion/deletion

miRNA microRNA

NGS next-generation sequencing

NHEJ non-homologous end joining

ODM oligo-directed mutagenesis

PTGS post-transcriptional gene silencing

RdDM RNA dependent DNA methylation

RNAi RNA interference

SDN site-directed nuclease, alternative name for SSN

siRNA small interfering RNA

SSN sequence-specific nuclease

TALEN transcription activator-like effector nuclease, a type of SSN

TGS transcriptional gene silencing

ZFN zinc finger nuclease, a type of SSN

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8. Annex

Table 1. *List of new techniques and/or developments in plant ("green") biotechnology as used in the interviews*.

Theme	Techniques	Mode of action/examples	Special
Genome editing	ODM oligo-directed mutagenesis	• inducing DNA-corrections by a homology-dependent repair mechanism using oligonucleotides with mismatches as template: indels, nucleotide substitutions	
Genome editing	Sequence-specific nucleases (SSNs): non-homologous end joining (NHEJ)	• inducing double strand breaks (DSBs) resulting in homologous-independent DNA repair by NHEJ using SSNs (ZFN, Meganuclease, TALENS, CRISPR-Cas9): indels, nucleotide substitutions	variant: non-transgenic genome editing (by delivery of protein or transient expression into cells)
Genome editing	Sequence-specific nucleases (SSNs): homologous recombination (HR)	• inducing double strand breaks (DSBs) resulting in homologous-driven repair by HR using SSNs: targeted gene insertion, allele replacement	
Plant transformation (new variants)	Plant transformation with transgenes not in end products	• Induced early flowering for accelerated breeding in fruit trees; • suppression of meiotic recombination for reverse (hybrid) breeding; • transgenic rootstocks	
Plant transformation (new variants)	Plant transformation introducing genes from cross-compatible species	• Cisgenesis: introduction of genes from same or cross-compatible species • Intragenesis: introduction of novel combinations of genes & promoters from same or cross-compatible species	
RNAi	Post-transcriptional gene silencing (PTGS)	• overexpression of gene-derived inverted repeats (dsRNA) for silencing gene expression: degradation of mRNA or translational repression directed by siRNAs or miRNAs	"Cross-kingdom RNAi": RNAi against pests and pathogens (host induced gene silencing HIGS) Special variants using transformed rootstock to deliver siRNA to non-GM scion

RNAi	Transcriptional gene silencing (TGS)	• gene silencing by promoter methylation induced by dsRNA from transgene subsequently removed from end product (RdDM)	Special variants: Induced using virus not carried over through meiosis. Mobile small RNAs moving between shoot & root (grafted plants, possibility of regenerating epigenetically changed plant from non-GM part)
Synthetic biology		• Synthetic chromosomes • Site-specific activators/repressors (targeted activation or repression of gene expression by artificial factors affecting transcription or inducing epigenetic changes using combinations of active domains with SSN DNA-binding domains) • Introduction of novel pathways; "plant as factory" (pharmaceuticals/antibodies) • Adapting CRISPR-Cas9 to targeting plant DNA viruses	

Method of delivery	Modifying gene expression without transformation	• Gene silencing by application of dsRNA:
		  ○ to plant (parts);
		  ○ to insects/pathogens
		• Agro-inoculation, VIGS
		• non-transgenic genome editing (by SSN delivery as protein or transient expression in cells)
			Special variant: early flowering by promoting or silencing gene expression through virus vector not transmitted to progeny

Other		• regenerating species hybrid from graft junction	

Questions per technique/concept:

1. What is your expectation for commercial applications/product development? What types of products can be expected?
   1.1. Short term
   1.2. Medium term. Do you expect any new developments/refinements? What could be the contribution of supporting technologies such as genomics/Next Generation Sequencing to new developments?
2. What do you see as drivers and what as barriers for application?
   2.1. Drivers
   2.2. Barriers

3. Finally, are any new developments/technologies missing in this list?