the plant journal

SEB Society for Experimental Biology

The Plant Journal (2014) **78,** 742–752 doi: 10.1111/tpj.12413

EMERGING TOOLS FOR SYNTHETIC BIOLOGY IN PLANTS

Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU

Frank Hartung* and Joachim Schiemann

Julius Kühn Institut, Federal Research Centre for Cultivated Plants, Institute for Biosafety in Plant Biotechnology, Erwin Baur Straße 27, D–06484 Quedlinburg, Germany

Received 11 September 2013; revised 4 December 2013; accepted 9 December 2013; published online 14 December 2013.
*For correspondence (email Frank.Hartung@jki.bund.de).

SUMMARY

Several new plant breeding techniques (NPBTs) have been developed during the last decade, and make it possible to precisely perform genome modifications in plants. The major problem, other than technical aspects, is the vagueness of regulation concerning these new techniques. Since the definition of eight NPBTs by a European expert group in 2007, there has been an ongoing debate on whether the resulting plants and their products are covered by GMO legislation. Obviously, cover by GMO legislation would severely hamper the use of NPBT, because genetically modified plants must pass a costly and time-consuming GMO approval procedure in the EU. In this review, we compare some of the NPBTs defined by the EU expert group with classical breeding techniques and conventional transgenic plants. The list of NPBTs may be shortened (or extended) during the international discussion process initiated by the Organization for Economic Co-operation and Development. From the scientific point of view, it may be argued that plants developed by NPBTs are often indistinguishable from classically bred plants and are not expected to possess higher risks for health and the environment. In light of the debate on the future regulation of NPBTs and the accumulated evidence on the biosafety of genetically modified plants that have been commercialized and risk-assessed worldwide, it may be suggested that plants modified by crop genetic improvement technologies, including genetic modification, NPBTs or other future techniques, should be evaluated according to the new trait and the resulting end product rather than the technique used to create the new plant variety.

Keywords: new plant breeding techniques, site-directed nucleases, GMO legislation, gene targeting, risk assessment, biosafety research.

INTRODUCTION

Plant breeding for improvement of plant-derived products used for human nutrition, feeding of domesticated animals or fibre production has been performed for thousands of years. In conventional breeding, this has been performed for a very long time, mainly by crosses of superior plants with other compatible plants to achieve more productive or pathogen-resistant plants, for example. With the realization that genes are the underlying elements determining qualitative or quantitative traits desired by breeders, the intention arose to mutate these genes specifically. As a result, traditional plant breeding has been accomplished over the last 60 years by mutagenesis using chemical compounds or irradiation application, followed by screening of mutation populations for the desirable traits. Traditional plant breeding techniques, including conventional mutagenesis, translocation breeding (Sears, 1956) and intergeneric crosses, are intrinsically very non-specific, as either a large genome part instead of a single gene is transferred by crossing, or thousands of nucleotides are mutated instead of the desired single one.

Since the mid 1990s, the classical repertoire of plant breeding techniques has been complemented by transgenic approaches to produce new plant varieties. These approaches were aimed at introduction of new resistance genes against plant pests and diseases (e.g. Bt-toxin producing maize) or herbicides on the one hand, and at modifying plants in order to produce desirable products (e.g. the starch-modified potato Amflora or 'Golden Rice') on the other hand. In comparison to conventional breeding,

production of transgenic plants may go beyond any natural crossing barrier, thereby increasing the available genetic variation, resulting in plants (or other organisms) that are not achievable by conventional breeding. Transgene technology has created great opportunities but at the same time raised a lot of questions concerning its possible impact on health and the environment. Parallel to the development of transgenic organisms, a rigorous public and political discussion took place in the 1990s, resulting in formulation of the Cartagena Protocol on Biosafety in 2000, which eventually came into force in 2003. This protocol primarily covers the intentional release of GMOs into the environment, and trans-boundary movement of GMOs for feed, food and production (Secretariat of the Convention on Biological Diversity, 2000). It also introduced the precautionary approach, and by 2013 had been signed by 166 countries. In the EU, non-contained production and propagation of genetically modified organisms is specifically regulated by Directive 2001/18/EC (European Parliament and European Council, 2001).

In this review, we provide an overview about the last two decades of market approval and cultivation of genetically modified plants (GMPs), and the great opportunities arising from plants developed by new breeding techniques that may or may not be regulated by GMO legislation.

CURRENT GMO LEGISLATION FRAMEWORK

Transgenic plants and the current GMO legislation

More than 20 years of research and regulation of genetically modified plants have passed since the first field trials in the EU took place. In 1990, Directive 90/219/EEC (for contained use of GMOs) and Directive 90/220/EEC (for deliberate release of GMOs) were adopted to protect human and animal health and the environment (The Council of the European Communities, 1990a,b). Therefore, the production, contained use and release of GMPs was covered by regulation in the EU not long after the first field trials were performed in 1986.

In 1996, commercial planting of GMPs started with approximately 1.7 million hectares worldwide, which had increased to 170 million hectares by 2012 (International Service for the Acquisition of Agri-biotech Applications, 2013). Thus, worldwide, the cultivation of GMPs has increased 100-fold in the last 17 years. In contrast, the total area of cultivation of GMPs in Europe was a mere 129 000 hectares in 2012 (International Service for the Acquisition of Agri-biotech Applications, 2013). This vast discrepancy between the EU and the rest of the world is mostly due to strong societal and political opposition against agro-food biotechnology (Devos *et al.*, 2012). This opposition is at least in part caused by rather negative media representation of agricultural biotechnology right from the start (Marks *et al.*, 2007). In contrast, the medical biotechnology resulting from GM processes was viewed positively by the media (Marks *et al.*, 2007). Most GMPs cultivated so far have been constructed using undirected first-generation transgenic procedures such as T–DNA integration via *Agrobacterium tumefaciens* and particle bombardment followed by tissue culture. All GMPs produced by transgenic procedures are regulated in the EU by Directive 2001/18/EC for cultivation and Regulation 1829/2003 for genetic modification (GM) food or feed (European Parliament and European Council, 2001; 2003).

In Directive 2001/18/EC, which replaced the older Directive 90/220/EEC, a GMO is defined as follows: 'Genetically modified organism (GMO) means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'. Annex IA of Directive 2011/18/EC provides a non-exhaustive list of known techniques that lead to a GMO, whereas annex IB lists known techniques that do not lead to a GMO.

Within the EU GMO legislation framework established in the 1990s, the precautionary approach was and still is a central point that was eventually adopted as a guide in Directive 2001/18/EC. Since the first field releases, a vast amount of safety research has been performed in Europe and elsewhere accompanying the GMPs used. This was of course necessary on the one hand for scientific reasons, as no data concerning GMPs in the environment existed, and on the other hand to address public concerns and fears at an early stage. The resulting huge amount of data has been repeatedly reviewed by various researchers, including very recently the Swiss National Science Foundation, assessing in total more than 2000 studies (Sanvido *et al.*, 2007; Qaim, 2009; Sehnal and Drobnik, 2009; Balazs *et al.*, 2011; Swiss National Science Foundation, 2012). Altogether, these investigations and research activities have shown that there is no valid evidence that GMPs have a greater adverse impact on health and the environment than any other crops developed by conventional plant breeding technologies. Therefore, from a scientific point of view, it is clear that the evaluated products of genetic modification crop technology are safe, and that there is no evidence-based indication of a general risk related to this technology *per se* (European Academies Science Advisory Council, 2013). The European Academies Science Advisory Council report comes to the conclusion that the process-based regulatory framework of GM (genetically modified) crops in the EU is not based on scientific evidence and is leading to asynchronous market approval in comparison with other countries. For example, the USA and Canada apply product-based regulation. Therefore, to avoid such disparity between trading countries now and in the future, and to place its regulatory framework on solid ground, the EU should aim for a new and more flexible regulatory system for agricultural biotechnology in the long-term. There is common agreement in the scientific community that

such an alternative regulatory system should focus on risk assessment and regulation of the trait and/or the product rather than the technology used to produce it (Morris and Spillaine, 2008; Podevin *et al.*, 2012; European Academies Science Advisory Council, 2013; Heap, 2013).

Current approval process

To approve a new GMP in the EU, the applicant must follow procedures that were established more than 10 years ago when only a limited amount of data concerning the impact of GMPs on human/animal health and the environment was available and evaluated. The complex authorization procedure(s) for placing a GMP on the market according to Regulation 1829/2003 and Directive 2001/18/EC is shown in Figure 1 (reprinted from Devos *et al.*, 2012). In cases of cultivation, the applicant must submit a notification/application dossier to the National Competent Authority of the Member State where the cultivation is planned. For non-food/feed applications, the National Competent Authority prepares an evaluation report and requests statements from the National Competent Authorities of other Member States via the EU Commission. If there are no objections, the responsible National Competent Authority takes a decision subject to the findings in the evaluation report. If objections by Member States remain (which is always the case), a route similar to that for food/feed use applications must be followed. For food/feed use applications, the dossier is passed to the European Food Safety Authority, which then informs the National Competent Authorities of the Member States and the EU Commission. The European Food Safety Authority considers its scientific opinion and collates the opinions of the National Competent Authorities. Based on this, a decision is drafted by the EU Commission, and representatives of the Member States then vote on the draft decision. If a qualified majority is not achieved, which frequently is the case, the EU Commission decides (see Figure 1). The risk assessment of GMPs is complex and differs with regard to the intended use for food/feed only or for cultivation in the EU (Craig *et al.*, 2008; Devos *et al.*, 2012).

The approval procedure following EU legislation is time-consuming (usually 4–6 years) and expensive (7–15 million euros) (Kalaitzandonakes *et al.*, 2007; McDougall, 2011; Tait and Barker, 2011; Anonymous, 2012). The process of

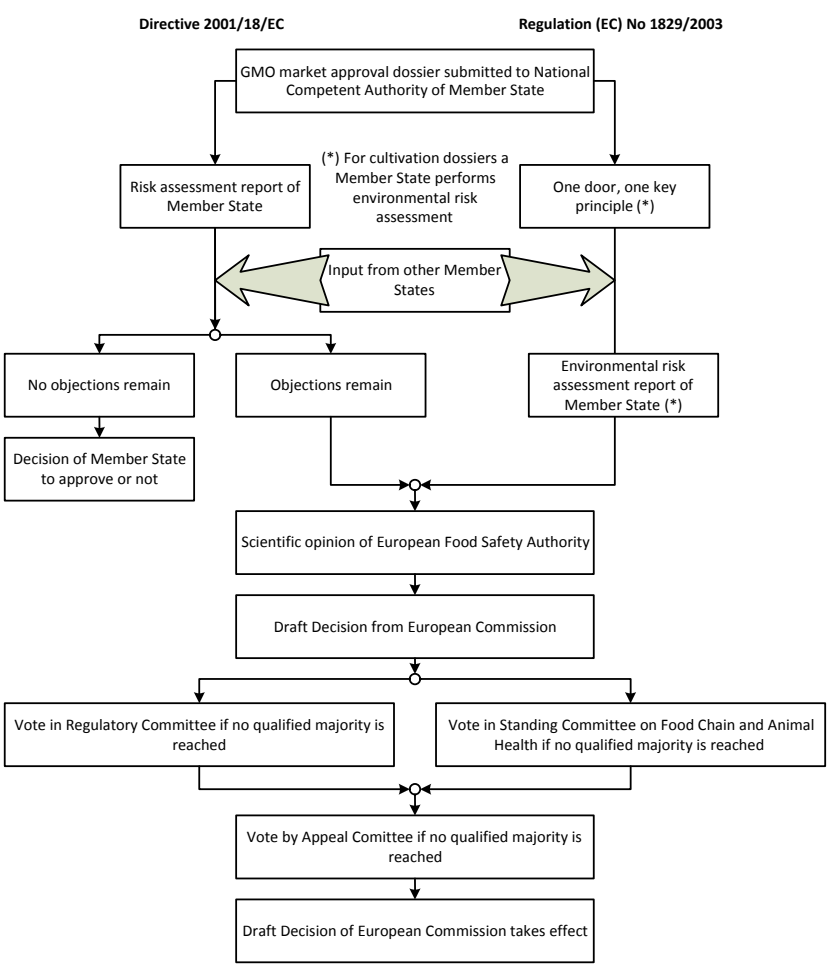

Figure 1. Authorization procedure for placing GM crops on the EU market.
The various steps that are necessary for approval of a new GMP following Directive 2001/18/EC and Regulation 1829/2003 are shown as a flow diagram. This figure has been adapted from Devos *et al.* (2012) with permission.

market approval of GMOs in the EU, and the societal and political rejection of agricultural biotechnology, currently prevent cultivation of new varieties derived through genetic engineering. Furthermore, even already approved events such as maize MON810 are not cultivated in several EU countries despite the absence of scientifically proven data on adverse effects of this maize on human and animal health or the environment. This situation is based on the safeguard clause that enables Member States to reject already approved GMOs in case of new scientific information about adverse effects. The safeguard clause may only be lifted by a decision of the Member States by qualified majority voting. In addition to the complex approval procedure, the continued destruction of experimental GMP field trials and the retreat of breeders resulted in 2011 in the lowest number of experimental field releases since 1992 (Marshall, 2012). Even safety research on transgenic crops is severely hampered (Gómez-Galera *et al.*, 2012). In summary, plant biotechnology for agricultural use is substantially more restricted in Europe than in other parts of the world (Tait and Barker, 2011; Sparrow *et al.*, 2013).

Taking the current situation described above into account, it is obvious that successful adoption of an agricultural biotechnological technique in the EU will only occur when the resulting organism does not fall under or is excluded from the GMO legislation. Coverage by the current GMO legislation will result in additional costs, a delay in commercialization and possibly negative perception. According to Heap (2013), the implications even go further: 'An EU regulatory position not based on sound science could create damaging knock-on effects for developing countries, which may depend on the EU for export markets or look to it for leadership in managing bioscience innovation. There is an ever-greater requirement for consistent, harmonized, evidence-based policy worldwide to enable synchronous technology development and trade'. Therefore, the European Academies Science Advisory Council suggests a radical reform of GMO legislation 'to be consistent with other international regulatory approaches and to learn from what has succeeded in regulation of innovation in other sectors' (European Academies Science Advisory Council, 2013).

NEW PLANT BREEDING TECHNIQUES

One of the main arguments concerning hazards that may arise from GMPs is uncontrolled integration of recombinant DNA into the genome. Such random integration may result in interrupted or de-regulated genes of the host plant, and therefore each new event must be evaluated with regard to changes in the host genome. For example, new fusion transcripts arising from interplay of the insertion and the genomic location are possible, and the potential for de-regulation of genes at the insertion locus makes it necessary to prove that the GMP is not altered in terms of its composition of nutritional elements and that it does not show new allergenic potential. However, all these effects may also occur using conventional breeding procedures such as crossing.

Since publication of Directive 2001/18/EC, a growing number of new techniques have been developed to enable more precise genetic modification of plants for research and precision breeding. As a positive side-effect, use of these techniques may avoid the problems of GMP market approval described above. The progress of precise genomic modification has been surprisingly rapid, but, for the new techniques, it is unclear whether either the technique itself or the organism produced by such techniques must be regulated by current GMO legislation. In total, eight techniques for which the regulation is unclear were named by the EU Commission and different National Competent Authorities in 2007. A New Techniques Working Group was founded due to a request by the National Competent Authorities of the EU for clarification of the legal state of these new techniques. The New Techniques Working Group completed its work in 2012, summarizing its findings as a report to the National Competent Authorities (New Techniques Working Group, 2012).

In 2009, the Directorate General for the Environment of the European Comission requested a study from the Joint Research Centre concerning the adoption, economic impact and possibility of detection of these new techniques. This study was performed in 2010/2011 under mandate from the Directorate General for Health and Consumers, leading to publication of a final report and a peer-reviewed article about new plant breeding techniques (NPBTs) (Lusser *et al.*, 2011, 2012).

The report by the New Techniques Working Group as well as the study by the Joint Research Centre focused primarily on the implications of new techniques for plant breeding, but the findings are also applicable to other organisms. The original list of these eight techniques and a short explanation is given below. For a more comprehensive description of these techniques, see Lusser *et al.* (2012) and the report of the New Techniques Working Group (2012).

NEW PLANT BREEDING TECHNIQUES THAT HAVE BEEN EVALUATED WITH RESPECT TO GMO LEGISLATION

Zinc finger nuclease (ZFN) technology

The term zinc finger nuclease is used in the original reports by Lusser *et al.* (2011) and the New Techniques Working Group (2012), but here and elsewhere is replaced by the term site-directed nuclease (SDN). Meganucleases and transcription activator-like effector nucleases (TALENs) possess a very similar mode of action to ZFNs, and all of these together may be covered by the term SDN (European Food Safety Authority Panel on Genetically Modified

Organisms, 2012a; Podevin *et al.*, 2013). The SDN technology has been divided into three sub-categories named SDN–1, -2 and -3, which are discussed below.

SDNs are DNA-binding and restriction proteins that are designed to recognize a specific DNA sequence. They either consist of a single protein chain that recognizes, binds and cuts a specific DNA sequence (meganucleases), or two proteins artificially connected by a peptide linker (ZFNs and TALENs). In the case of ZFNs/TALENs, the protein responsible for DNA recognition and binding may be variably designed for different specific DNA sequences, but the fused nuclease protein is usually *Fok*I, which cuts any DNA sequence non-specifically. ZFNs and TALENs act as heterodimers; thus, for a successful reaction, two genes must be expressed in the cell. SDNs may be used for targeted genome mutation, including editing, insertion, deletion or replacement of genes, and stacking of molecular traits (Shukla *et al.*, 2009; Townsend *et al.*, 2009; Osakabe *et al.*, 2010; Petolino *et al.*, 2010; Fauser *et al.*, 2012; D'Halluin *et al.*, 2013). At the mandate of the EU Commission, the European Food Safety Authority published an opinion addressing the safety assessment of plants developed using zinc finger nuclease 3 and other site-directed nucleases with similar function (European Food Safety Authority Panel on Genetically Modified Organisms, 2012a).

Over the last 2 years, details of the CRISPR/Cas9 nuclease (clustered regularly interspaced short palindromic repeats), a new kind of SDN, have emerged (Jinek *et al.*, 2012; Wei *et al.*, 2013). In this system, the Cas9 nuclease is guided to a genomic sequence by a specific guide RNA. This guide RNA binding principle is technically different from the above described mode of action, but nevertheless involves a site-directed nuclease.

Oligonucleotide-directed mutagenesis

The basis of oligonucleotide-directed mutagenesis (ODM) is a modified DNA or DNA/RNA oligonucleotide of 20–100 nucleotides that is delivered into the cell by suitable methods. The sequence of the modified oligonucleotide is homologous to a genomic sequence, but differs in one or a few nucleotides. Therefore, after binding of the homologous genomic sequence, a mismatch pairing is created that is corrected by the repair system of the host cell, leading to specific mutations if the sequence of the oligonucleotide is used as the template for synthesis of the new DNA during the repair process. ODM may be used for targeted genome editing, e.g. to induce herbicide resistance by point mutation (Beetham *et al.*, 1999; Zhu *et al.*, 2000; Oh and May, 2001).

Cisgenesis and intragenesis

Cisgenesis/intragenesis involve transfer of an intact gene or a DNA fragment between organisms of the same species or from a cross-compatible species (Jacobsen and Schouten, 2007). In the case of cisgenesis, the transferred gene is unchanged, whereas, for intragenesis, parts of a gene (e.g. regulatory elements) may be transferred. Cisgenesis may lead to a new organism that is indistinguishable from a conventional cross. Intragenesis always leads to an organism that is not obtainable by conventional crosses. The European Food Safety Authority recently published an opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis (European Food Safety Authority Panel on Genetically Modified Organisms, 2012b).

RNA-dependent DNA methylation

The RNA-dependent DNA methylation (RdDM) method enables modified gene expression by transcriptional gene silencing or promoter methylation without changing the genomic sequence. The methylation patterns are induced by double-stranded RNAs that are processed by various host enzymes of the RdDM machinery, including polymerases IV and V, Argonaute proteins and cytosine methyl transferases (Mahfouz, 2010). The epigenetic changes may be inherited and stable for at least a few generations. RdDM may be utilized to modify the expression of one or more genes.

Grafting on GM rootstock

Grafting itself is a classical breeding method in which two plants with different phenotypes are combined by physical attachment. When the lower part, the rootstock, is taken from a transgenic plant and the upper part, the scion, is from a conventional plant, the resulting leaves, stems, seeds and fruits do not carry transgenic DNA. As an example, this method may be used for expression of interfering RNAs (or RdDM) in the rootstock; these are systemically transported and may lead to transient or inheritable silencing of genes in the scion. Thus, the resulting seeds, fruits or offspring from such a scion do not contain any DNA of transgenic origin, whereas adventitious shoots regenerating from callus or rootstock may carry such transgenic DNA (Stegemann and Bock, 2009; Nagel *et al.*, 2010; Lusser *et al.*, 2011).

Reverse breeding

Reverse breeding is a technique that relies on suppression of meiotic recombination during propagation of an elite hybrid plant. The meiotic recombination is suppressed by silencing or knockout of genes that are essential for meiotic crossover but leave the chromosomes intact, e.g. SPO11 (Hartung *et al.*, 2007; Wijnker and de Jong, 2008; Dirks *et al.*, 2009; Wijnker *et al.*, 2012). Therefore, the allelic chromosomes are not paired during meiosis and are distributed by random segregation only. Viable microspores containing in some cases by chance a complete haploid

chromosomal set are then converted to a double haploid state using the doubled haploid technique (Forster *et al.*, 2007). As the chromosomes segregate randomly, reverse breeding is applicable only for plants that possess a small number of chromosomes (Dirks *et al.*, 2009). The advantage of reverse breeding is that elite hybrid plants with unknown parents may be used directly to reconstruct the homozygous parental plants *de novo*, which is essential to maintain the hybrid line.

Agro-infiltration

Agro-infiltration means that a plant tissue is infiltrated with an *Agrobacterium* suspension. The bacteria contain the genes to be expressed in the plant. Therefore, the desired genes are expressed locally and only transiently in the plant, producing recombinant proteins at high levels, for example (Vezina *et al.*, 2009). This technique may be used for localized expression in the infiltrated area only (*sensu stricto*), for systemic expression following inoculation (agro-inoculation) or for germline transformation (floral-dip method; Clough and Bent, 1998). In the two former cases, the inoculated plants are not propagated, whereas the floral-dip method usually leads to transgenic offspring.

Synthetic genomics

One of the main goals of synthetic genomics is the design of artificial biological systems to study the prerequisites of life and to create new production platforms (Benner and Sismour, 2005). Initially, a complete bacterial genome was synthesized and transformed into a yeast cell (Benders *et al.*, 2010). After propagation, this artificial genome may be transplanted into empty bacterial cells, giving rise to a new synthetically constructed organism (Lartigue *et al.*, 2007, 2009).

COMPARISON OF THE TECHNICAL STATE OF NEW PLANT BREEDING TECHNIQUES

In our view, the eight techniques that are under consideration for regulation by GMO legislation are quite different concerning their technical ability to modify the genome or gene expression of an organism. Therefore, we have arranged them into three groups, complemented by a fourth group that comprises high-throughput techniques. The high-throughput techniques are not subject to GMO legislation at all because they are purely analytical techniques (see Figure 2).

Basic techniques (gene modification techniques)

This group includes SDN technology and ODM (both of which introduce genetic modifications) and RdDM (which introduces epigenetic modifications), because these techniques are used for modification of an existing DNA sequence in a plant either by mutation, insertion/deletion and gene replacement, or by stable silencing of a gene body or the promoter (or other regulatory elements). These techniques provide appropriate tools such as designed SDNs (with or without accompanying homologous DNA), an oligonucleotide for ODM, or an RNA silencing construct designed for transient or stable expression in the host cell to alter its genome sequence. These various constructs create recombinant DNA when they are integrated into the genome; they must be transferred to the plant first to exert their effect.

Transfer techniques

This group comprises grafting on GM rootstock and agro-infiltration (transient and stable), as these techniques have

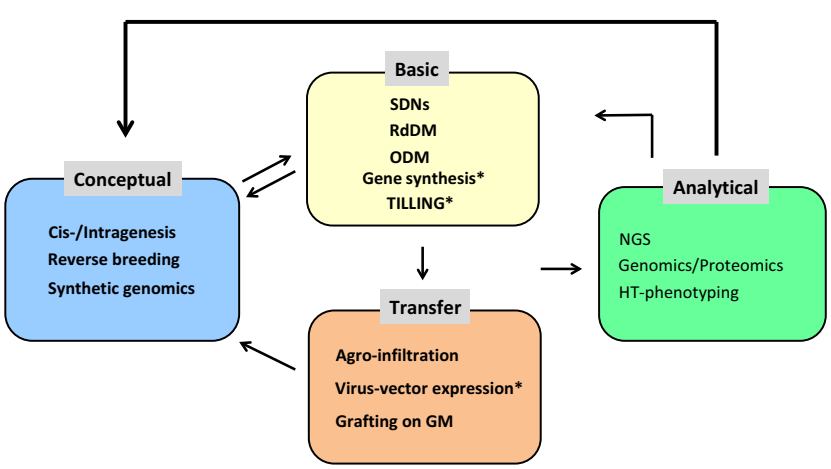

Figure 2. New plant breeding techniques grouped by their possible effects.
The conceptual techniques are new ideas rather than just simple procedures. They may be performed using a combination of basic techniques that provide the constructs and enzymes to modify or synthesize genomic DNA. The resulting constructs may be transferred into the plant cell by various transfer techniques. The development of any new technique may influence other groups, either by providing background data for intended gene modifications (analytical and conceptual) or the procedures necessary to modify the genome (basic and transfer). Gene synthesis, TILLING and virus vector expression do not fall under the scope of GMO legislation, nor do the analytical techniques. Abbreviations: GM, genetically modified; HT, high-throughput; NGS, next-generation sequencing; ODM, oligo-directed mutagenesis; RdDM, RNA-dependent DNA methylation; SDNs, site-directed nucleases; TILLING, targeted induced local lesions in genomes.

been developed to introduce and express a designed construct that performs its function in the whole target organism or specific tissues/cells only.

Conceptual 'techniques'

This group comprises cisgenesis/intragenesis, reverse breeding and synthetic genomics. In principle, conventional transgenesis may also be considered as a conceptual technique. These techniques are not techniques *per se*, but rather provide a conceptual definition regarding what is altered in the organism of choice by various specific techniques. Transfer of a designed construct or suppression of meiotic recombination and the genetic modification itself occur via transfer and genetic modification techniques.

Analytical techniques

The fourth group comprises modern high-throughput analysis techniques such as next-generation sequencing, genomics, transcriptomics and proteomics (and other -omics techniques), as well as high-throughput phenotyping. These techniques are not within the scope of GMO legislation as they are not aimed at modification of an organism. Nevertheless, these techniques and their improvements are necessary to prepare the ground for basic and conceptual techniques as sufficient sequence information regarding a given locus, chromosome or even genome is required to design a specific SDN, ODM or RdDM (Figure 2).

Other authors have described other groupings (Podevin *et al.*, 2012; Lusser and Davies, 2013), focused in such a way as to alleviate a policy decision whether a group as a whole may be subject to GMO legislation. We decided to be more pragmatic and therefore based our grouping on the impact of the technique in the developmental or production process of a new variety, showing that there is no need for GMO-specific regulation of a group of respective techniques *per se*.

COMPARISON OF CLASSICAL BREEDING TECHNIQUES AND CONVENTIONAL TRANSGENIC PLANTS WITH NPBTS

Here we focus on gene modification techniques used for targeting of genes located in the nuclear genome, an objective that a few years ago was virtually unfeasible in plants at all, except *Physcomitrella patens* (Strepp *et al.*, 1998). Mutation of plant genes used in conventional breeding is a technique that is excluded from GM legislation by Annex IB of Directive 2001/18/EC. It is achieved by either irradiation, application of chemicals, or somaclonal variation (as a consequence of tissue culture technique). These methods are used to induce point mutations or small insertions/deletions. To induce larger insertions or even combine chromosomes from sexually incompatible plants, crosses, wide crosses (including embryo rescue and somatic hybridization) and translocation breeding are applied (Sears, 1956; Van Eijk *et al.*, 1991; Liu *et al.*, 2005; Miyajima, 2006). The methods used in mutation breeding have been in use since the 1950s and are excluded from GMO legislation, because such mutations also occur without human intervention but with a much lower frequency. The occurrence of mutations is considered to be a natural process, commonly used by breeders to exploit natural variation, that is simply boosted by the applied technique. Up to now, more than 3200 officially released cultivars are known to have been obtained by mutation breeding, among them more than 600 cultivated lines of wheat, rice and maize (http://mvgs.iaea.org).

The application of SDN–1 or ODM produces similar mutations to those ones that may occur in a natural process or by mutation breeding, but in a much more specific way (de Pater *et al.*, 2009; Puchta and Hohn, 2010; Curtin *et al.*, 2011; Tzfira *et al.*, 2012). Instead of the thousands of mutations per genome induced by mutation breeding, SDN–1 or ODM techniques result in only very few mutations other than the desired ones. These off-target mutations are due to non-specific binding of the oligonucleotide or the binding part of the SDN. With regard to the SDNs, highly specific cleavage may be achieved either by improvement of the binding specificity, as more specific binding strongly reduces the off-target effects, or by *in vitro* pre-selection of the best SDN (Cornu *et al.*, 2008; Pattanayak *et al.*, 2012; Sander *et al.*, 2013). In principle, SDN–1 and ODM are similar to mutation breeding, but are much more specific and straightforward. Therefore, there is a general tendency to exclude SDN–1 and ODM from GMO legislation (Lusser and Davies, 2013). In SDN–2, a template DNA (usually called the donor) is added, which is homeologous (very similar but not identical) to the genomic sequence to be modified (Lusser *et al.*, 2011). The sequence of the added donor DNA may be used as a template, and thereby copied via replication into the genome and replace the original sequence. This SDN–2 process is similar to a natural DNA repair process that may easily occur between different alleles in a genome, for example. Consequently, regulation of SDN–2 under GMO legislation is unlikely.

The SDN–3 technique is aimed at targeted alteration of a specific genomic sequence by replacement and integration of a different gene, which may be cis-, intra- or transgenic. For this purpose, which is generally known as 'gene targeting', long stretches of homologous DNA are added. This homologous DNA flanks the area of the genome to be modified. It does not necessarily precisely surround the double-strand break but may be located in the vicinity (within some hundred bases up to kilobases), as processing of the double-strand break may extend over a long distance (Zhu *et al.*, 2008). The combination of a double-strand break and the homologous DNA enhances

homologous recombination, and, in the case of successful repair by homologous recombination, the sequence between the flanking homologous DNA is exchanged for the sequence encoded on the template DNA. The whole process of homologous recombination-dependent double-strand break repair may be used for alteration, excision or integration of new genes (D'Halluin *et al.*, 2008; Cai *et al.*, 2009; Shukla *et al.*, 2009). The SDN–3 technique is partially comparable to the crosses and wide crosses used in conventional breeding. However, for integration of genes from other organisms than plants, the result of SDN–3 is not comparable to natural plant breeding processes, and therefore this technique will be most probably regulated in the same way as transgenic plants are today. Compared to conventional crossing techniques, SDN–3 is several orders of magnitude more precise, and most of the time-consuming steps necessary to get rid of unwanted sequences and to minimize side-effects are not necessary.

The RdDM technique makes use of transient expression of a specifically designed RNA molecule that is processed by the natural system of RdDM that already exists in plant cells (Wassenegger, 2000; Aufsatz *et al.*, 2002). The double-stranded or hairpin RNA molecule is processed by the natural cellular silencing machinery, leading to methylation of histones and subsequent methylation of the DNA sequence bound by these histones. This is solely an epigenetic change, as the original DNA sequence is not mutated. The methylation state of the DNA may be stable throughout meiosis for several generations at least (Mahfouz, 2010). Epigenetic modifications of the genome occur in nature without any intervention of humans. In every plant analysed so far, epigenetic modifications induced by various environmental factors such as drought, heat, flooding or pathogen defence mechanisms (Grativol *et al.*, 2012; Silveira *et al.*, 2013) have been detected. The only factor determining whether a plant treated by the RdDM technique is transgenic or not is possible integration of the RNA (after reverse transcription) or vector sequences into the genome. If such sequences are not integrated, the resulting plant does not contain any recombinant DNA, and is therefore a non-transgenic plant. Furthermore, such a plant is not even mutated by the common definition of a mutation as the genomic nucleotide sequence is not altered.

Taking the above-mentioned considerations into account, in principle the SDN–1, -2 and ODM techniques mimic a natural mutation, whereas the RdDM technique provokes an epigenetic change in the DNA sequence similar to those that occur naturally. The only reason to consider plants produced by these new genomic modification techniques as transgenic is integration of foreign DNA into the genomic DNA, resulting in a recombinant DNA that could not occur by natural processes. As described above, this is only true in some cases of SDN–3 or if recombinant DNA arises during RdDM or SDN–1 and -2 (stable integration). However, in the latter case, in which the expression construct for SDN–1 and -2 or RdDM is stably integrated in order to fulfil its function, these constructs may be removed after their successful action by back-crossing and segregation to obtain plants that no longer contain the recombinant DNA.

Reflecting on the above considerations in light of the current GMO legislation, a new plant variety may be regulated as a GMP if its development involves a genetic modification step (Schaart and Visser, 2009; Tait and Barker, 2011). From the scientific point of view, there is no reason why a plant should be regarded as transgenic when it does not contain recombinant DNA. To avoid debate about offspring of transgenic plants, it is possible to express the construct (SDN, ODM or RdDM) only transiently, or to integrate it only in the rootstock part in case of grafting, as there is no evidence of recombinant DNA transfer into the scion except for the callus region at the interface (D'Halluin *et al.*, 2008; Cai *et al.*, 2009; Stegemann and Bock, 2009). The transient expression and successful action of such a construct *in planta* has been demonstrated using a vector system derived from tobacco rattle virus for successful ZFN-directed repair of a GUS gene in petunia and tobacco (*Petunia hybrida* and *Nicotiana tabacum*) (Marton *et al.*, 2010). As no template DNA was added in this reaction, the transiently expressed ZFNs used in this experiment cleaved the DNA, which then was repaired by non-homologous end joining, leading to functional restoration of the GUS gene in some cases (Marton *et al.*, 2010). In general, transient expression of SDNs in plants by viral vectors possesses the great advantage that the vectors may travel from cell to cell, leading to systemic expression of the recombinant protein without integration of recombinant DNA (Lico *et al.*, 2008; Vainstein *et al.*, 2011).

Proof-of-concept experiments to construct plants using SDNs that are not SDN–1 or -2 techniques have involved integration of a so-called 'landing platform' by conventional transgenesis into the genome of Arabidopsis (Fauser *et al.*, 2012). These plants are transgenic as they contain the designed landing platform construct, but they provide basic lines into which any new DNA may be integrated at this artificial but well-characterized locus. This means that the same already characterized locus is targeted in any subsequent plant produced by the SDN–3 technique, avoiding new potential side-effects.

Further, combinations of these new techniques may be developed, e.g. one may express an SDN construct or another gene modification construct using the agro-infiltration technique to ensure it is expressed only transiently without integration of foreign DNA. This agro-infiltration technique has been used for transient expression of chicken α–interferon in *Lactuca sativa* (Song *et al.*, 2008).

CONCLUSION AND PERSPECTIVES

The regulatory process for market approval described above, and the list of eight NPBTs, mainly reflects the European situation. In other countries around the world, crop genetic improvement technologies including GM technology are assessed in different ways (Waltz, 2012; Lusser and Davies, 2013). The growing number of crop genetic improvement technologies accompanied by elaborate transient transfer and expression techniques, as well as modern concepts such as synthetic genomics or reverse breeding, aided by sophisticated high-throughput-analytical techniques, provides a set of superior tools to quickly and precisely alter the genomic sequences of plants. Using these techniques, potential adverse effects are even less likely than in conventional transgenic plants or plants resulting from conventional breeding. The combination of various new techniques will allow precise genetic modification, resulting in plants that harbour as little recombinant DNA as possible or none at all.

During more than 20 years of biosafety research on conventional transgenic plants, no adverse effects on health or the environment have been attributed to recombinant DNA technology *per se* (Swiss National Science Foundation, 2012). Nevertheless, the decision whether a modified plant is regarded as transgenic or not is based on the techniques used to produce it. The NPBTs (Figure 2) are very diverse with regard to their technical impact, and the techniques will most probably be used in various combinations to produce new plant varieties. If one technique of a combination of techniques were classified as GM technology, the whole process and the resulting plant may be regulated as a GMO. This may result in production of new plant varieties by classical breeding techniques, conventional transgenic techniques or new plant breeding techniques that may be virtually identical but will be handled as a GMP in some cases but not in others. For example, the conceptual technique of cisgenesis may lead to a plant in which a given resistance gene is replaced by a resistance gene from a cross-compatible plant at exactly the same locus, using an SDN and homologous DNA for gene targeting (basic technique) that is expressed only transiently by agro-inoculation (transfer technique). This virtual experiment (which is most probably already a reality in some laboratories) utilizes techniques that, according to the current GMO legislation, have to be regulated.

How may such an experiment be evaluated? Three new techniques are combined in a single experiment. According to the New Techniques Working Group, cisgenesis may fall under GMO legislation, whereas the transfer technique results in only transient expression of recombinant DNA and may not fall under GMO legislation. The gene targeting technique itself is SDN–3 and most probably will be regulated. The decision process is questionable because the resulting plant is indistinguishable from a cross between the crop plant and a cross-compatible plant in which exactly this recombination occurred by chance. Even more intriguing, there are no detection methods available to ascertain which plant was produced by which technology, as there no vector sequences or resistance genes are present. Furthermore, the resulting plant will be more convenient, as no linkage drag or other side-effects occur, because only the gene of interest is recombined in this case, leading to a precisely designed new plant variety.

This example and further applications that are now possible using the NPBTs show clearly that common regulation based solely on the technique used is not evidence-based and is not appropriate for NPBTs. As stated above, there is no scientific reason to classify a plant as a transgenic organism, resulting in extensive time-consuming and expensive steps for market approval, if there is no foreign recombinant DNA in its genome. In our opinion the scientific community is requested to provide advice to policy makers on how to handle NPBTs and their possible hazards in a scientifically sound way. Therefore, in accordance with other reports and statements, we argue for a paradigm shift towards a more flexible and product-based GMO legislation focused on the potential hazards of the resulting end product rather than the process leading to it (Morris and Spillaine, 2008; Breyer *et al.*, 2009; Podevin *et al.*, 2012; European Academies Science Advisory Council, 2013; Heap, 2013). This would be based strongly on the accumulated scientific evidence with respect to the biosafety of GMOs (Swiss National Science Foundation, 2012). Based on the history of GMO legislation in the EU, it is not expected that such a paradigm shift will occur short-term or even medium-term. Therefore, in the near future, we urgently require more pragmatic handling of the NPBTs, such that modified plants that do not contain recombinant DNA are exempt from regulation (e.g. inclusion in Annex IB of Directive 2001/18/EC), and those containing recombinant DNA (which is not a hazard *per se*) are de-regulated in some way as unintended side-effects are expected to be lower than in first-generation transgenic plants (European Food Safety Authority Panel on Genetically Modified Organisms, 2012b; Podevin *et al.*, 2012; Pauwels *et al.*, 2013).

Without a change in risk assessment and regulation, Europe will face remarkable disadvantages regarding development and propagation of plants produced by NPBTs. The need for de-regulation of such plants was recently emphasized by Heap (2013): 'Confirmation by the EU that targeted techniques that leave no foreign DNA behind do not fall under the scope of GM legislation would give considerable support to agricultural innovation in Europe'. Without such a confirmation, 'there is the risk that scientists and companies will move elsewhere, accelerating the negative impact on the science base and on Europe's competitiveness'

(Heap, 2013). Summarizing the recent discussions on risk assessment and regulation of crop genetic improvement technologies, we need a change regarding the regulation such that 'the objective [is] to regulate the product and not the technology that produces it' (Heap, 2013).

ACKNOWLEDGEMENTS

We thank Angelika Ziegler (Julius Kuehn Institut, Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostic, Quedlinburg, Germany) and Ralf Wilhelm (Julius Kuehn Institut, Federal Research Centre for Cultivated Plants, Institute for Biosafety in Plant Biotechnology, Quedlinburg, Germany) for thorough reading of the manuscript.

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