AgBioForum, 17(2): 183-190. ©2014 AgBioForum.

Use of Novel Techniques in Plant Breeding and Practical Consequences Concerning Detection, Traceability, Labeling, and Risk Assessment

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Genetically modified plants (GMP) are regulated by comprehensive EU legislation. Cisgenesis, intragenesis, oligo-directed mutagenesis (ODM), zinc-finger nucleases (ZFN), and agroinfiltration were examined concerning detection, traceability, labeling, and risk assessment. Similar transformation methods—as in transgenesis using genetic elements from same or cross-compatible plant species—characterize cisgenesis and intragenesis. Targeting the genome with ODM or ZFN minimizes unintended effects, but current data indicate limited efficiency and specificity; modifications are similar to those occurring during traditional plant breeding. The characteristics of plants produced by these techniques affect detection in the supply chains. Detection is possible when the target site is known, except for agroinfiltration that aims at transient expression of a gene/trait. The basis to assess potential risks arising from relevant plants and derived food and feed is similar to that of GMP. Depending on the specifics of the plant under investigation, data requirements for regulators may be reconsidered case-by-case.

Key words: agroinfiltration, cisgenesis, food/feed safety, gene targeting, genetically modified plants, new breeding techniques, oligonucleotide-directed mutagenesis, protein engineering, risk assessment, zinc fingers.

Introduction

The technical and scientific progress in plant breeding and plant transformation technologies together with novel construct designs results in rapidly evolving new concepts and breaking innovations beyond the usual approaches. They open up questions of fundamental and practical relevance to regulators, stakeholders, and consumers. To provide the basis for clarification whether the application of new plant-breeding techniques results in plants defined as genetically modified organisms (GMOs) according to the relevant EU legislation, the European Commission (EC) has launched a number of activities addressing, *inter alia*, the regulatory framework and the safety assessment of new plant-breeding techniques. The EU regulatory definitions of GMOs according to Directive 2001/18/EC (EC, 2001, p. 18) foresee the exemption of some genetic modification techniques/methods such as mutagenesis or "cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods."

To date, no products developed through the application of new techniques have been placed on the European market. How soon such products will be commercialized depends on many factors, including legislative decisions on the European level. Some new techniques have already been adopted by the breeding sector in their research and development and are at advanced development stages.

The new techniques under investigation (cisgenesis, intragenesis, oligo-directed mutagenesis [ODM], zinc-finger nucleases [ZFN], and agroinfiltration) can supplement traditional plant-breeding techniques. In some cases, only direct gene insertion or targeted mutagenesis offer the possibility to achieve a desired trait in a given plant. This is particularly true for plants with long lifespans like trees, in which resistance breeding may be accelerated by the new techniques. In addition, the properties of the products (e.g., taste, appearance) are left unchanged, as no additional changes to the genome are expected.

In order to use the novel techniques successfully, the gene(s) encoding for the desired traits have to be fully characterized. Consequently, monogenic traits (e.g., monogenic resistance) are ideal candidates for employing cisgenic methods. However, it has to be taken into account that the combination of multiple resistance genes contributes to the prolonged maintenance of the resistance. Many important traits are the result of genetic interaction. Therefore, frequently gene stacking

is necessary, either by crossing or by transformation using multigene cassettes.

Transgenesis may extend the gene pool of the recipient species, and it frequently creates completely artificial traits. In contrast, cisgenesis involves only genes from the plant itself or from a close relative capable of sexual hybridization; the genomic region containing the gene of interest is left contiguous, including its introns and all regulatory elements (promoter and terminator regions). According to the definition of the cisgenic concept (Schouten, Krens, & Jacobsen, 2006), no alteration whatsoever of the native status of a plant gene is permitted. Whereas intragenesis similarly uses sequences from inside the sexually compatible gene pool, it allows designing traits by rearranging genetic elements *in vitro* (Rommens, Haring, Swords, Davies & Belknap, 2007). Thus, gene silencing approaches may be used that cannot be attempted in a targeted manner with cisgenic methods. Importantly, in both concepts, additional sequences and foreign genes such as selection markers and vector-backbone genes should be absent or, alternatively, eliminated from either the primary transformants or their progeny.

At present, for all techniques, the same transformation methods as in transgenesis are applied, resulting in similar possibilities concerning the occurrence of unintended effects. To overcome transformation and/or regeneration-induced negative side effects, rigorous selection and subsequent breeding programs similar to traditional strategies are necessary. Cisgenic plants are commonly produced through *Agrobacterium*-mediated transformation as the method of choice (Lusser, Parisi, Plan & Rodríguez-Cerezo, 2011).

In site-directed mutagenesis, endogenous genes are specifically targeted. Several methods generating targeted, site-directed mutations within genomes have been developed. Among these, oligonucleotide-directed mutagenesis (ODM), which is known under various names, has been used extensively. Upon delivery, the oligonucleotides are degraded by the cell within hours, resulting in transient exposure of the cells to the oligo. Whereas the inducing molecules are not heritable, they lead to heritable alterations in the genome. A more recently developed technique based on the use of zinc-finger nucleases (ZFN) causes the site-directed mutagenesis of single or few nucleotides in a plant genome (ZFN-1, ZFN-2); the ZFN technique may also be designed to allow for the site-directed insertion of longer stretches of DNA (ZFN-3). ODM, ZFN-1, and ZFN-2 target homologous sequences in the genome and are therefore expected to be highly specific. They may lead to the introduction of new genetic information, including point mutations, the reversal of an existing mutation, or deletions; it is also possible to silence genes by these techniques.

Site-directed mutagenesis may be applied to any given plant if the sequence to be modified—including the effect of the sequence alteration—is known. In contrast to genome modification methods like transgenesis, cisgenesis, or intragenesis, no DNA—neither from sexually compatible nor from incompatible species—is inserted. Several patents for ODM have been filed, and the technology is commercially available. Plants produced using ODM are expected on the market within the next few years. The list of crops modified by ODM-based techniques is continuously increasing, as companies have started cooperating on traits in a number of crops. Current developments focus on herbicide tolerance and, to a lesser extent, on resistance against various pests. In any case, it is necessary to have at hand an efficient selection system after the genomic alteration. ZFN-technologies are a relatively new area of research; therefore, in the medium-term, considerable progress is to be expected. However, it is not feasible to anticipate commercial developments in the next few years.

In addition to the widely employed stable transformation of plants by *Agrobacterium*-mediated transformation, *Agrobacterium* may also be used to express genes transiently. Several factors influence the duration of gene expression; however, in all cases the time of expression is limited. Agroinoculation and agroinfiltration may be applied for molecular farming used to produce valuable proteins like plant-made pharmaceuticals in plants, the analysis of gene function (e.g., in plant-pathogen interactions), stress tolerance studies, and resistance breeding. Integration into the plant genome is not expected but could happen in rare cases.

All new techniques under investigation may be combined with cisgenic approaches; however, the potential combination is governed by the breeding goal and may be of limited usefulness. ZFN-3 is a promising technique that potentially can be combined with cisgenesis on a large scale—provided sufficient specificity and efficiency. The combination of the two techniques overcomes the random integration of genes associated with current transformation technologies. Consequently, it might potentially hamper the event-specific detectability of the genetic modification, as the newly inserted sequence may not be sufficiently diverse from the replaced endogenous one.

Detection

Only unambiguous detectability of GMOs along the food production chain gives the consumer the possibility to make a qualified choice. Detection of GMOs may be confounded by a lack of suitable methods (a technical deficiency) and/or constraints on what information regulators can require (a regulatory deficiency; Sustainability Council of New Zealand, 2011). Regulatory requirements play an important role in determining whether GMOs are readily identifiable and traceable (EC, 2013). For the detection of any authorized GMO, a method validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) and certified reference material has to be available. This is the case if an applicant has launched the application procedure for authorization of a GMO within the EU; lab-based quantification methods as foreseen in the relevant legislation are then available.

To date, GMOs have been engineered through the stable integration of a recombinant genetic cassette into the genome of a recipient organism. In the past, detectable elements like promoters and terminators were interchangeable and suitable for a number of GMO events. Novel GMOs frequently carry unique combinations of genetic elements that have to be disclosed in order to develop an appropriate detection method. However, due to the unique combination of the genomic DNA sequences and the randomly inserted sequence, it is straightforward to develop an event-specific detection method making use of this characteristic. Similarly, cisgenic and intragenic modifications may be detected by applying standard methods. Like for all GM methods leading to stable integration of additional sequences, detection of stable genomic alterations caused by *Agrobacterium*-mediated transformation is possible by means of current standard molecular detection methods (real-time PCR) if adequate information on the alteration is available. In addition, the newly expressed protein may be detected by protein-based methods, as both primary transformants and their progeny express the desired product.

Given the precondition that plants are subject to GMO or similar regulations, specifications on the modification in the plant genome are available. If this is not the case, no information on the DNA modification is given. Similar to unauthorized GMOs, no official detection method is available due to the lack of an authorization process, and introduced genetic elements and induced modifications cannot be traced. Consequently, the novel modifications cannot be detected with existing, already validated methods, and event-/mutation-specific detection methods are necessary.

Plants developed through new techniques frequently display characteristics that affect laboratory testing and detection in the supply chains. Regardless of the applied technique, prior knowledge on the modification is the key factor for rendering its detection possible. The routine detection of relevant modifications requires molecular information on the event, which will be necessary to allow for its detection in the test laboratory. In order to reliably detect cisgenic modifications in plants using currently available methods in routine analysis, information on the alteration (i.e., the cisgene itself) as well as on the flanking regions of its insertion site has to be available. Provided that the site of the genetic modification in the genome is known, detection methods may generally be developed for all plants derived from the use of novel techniques, and unmodified plants may clearly be distinguished from modified ones. Also, unintentional mutations can be detected, at least by direct sequencing. These may be a result of evolution but also a by-product of intended modifications of the plant genome. Despite detection of even small alterations in the genome *per se* is possible provided that the necessary information is available, both traceability and labeling are seriously hampered.

For plants produced by ODM and ZFN that harbor point mutations, a clear additional challenge is to identify the source of the genetic modification. Although the detection of small mutations in the genome is possible with current methods, their identification (i.e., the clear verification of their origin) is not. Generally, they cannot be distinguished from those occurring spontaneously. In this context, it is essential to distinguish between detection and identification. Detecting a change means the possibility of determining the existence of an alteration to the genome in comparison to conventional counterparts or natural variants. The identification of a change implies the possibility of determining that this particular change in the DNA has been intentionally introduced by targeted mutation techniques (e.g., ODM or ZFN). If it is not possible to identify the origin of the particular modification in the DNA, i.e., whether it is the result of conventional mutagenesis, a spontaneous mutation, or has been introduced intentionally through ODM or ZFN techniques, the development of an unequivocal quantification method can be difficult.

Novel transformation technologies potentially redefine the types of analytical target. Cisgenic modifications of plants may result in products/seed, etc., in

which the detection of the modification is hampered by the nature of the modification, e.g. the insertion of additional copies of genes already present in the plant genome. Hence, the presence of a similar protein prior to a cisgenic or intragenic modification frequently excludes the use of protein-based detection methods. Whereas cisgenic modifications can be clearly detected on the DNA-level, provided that the relevant information is available, the use of protein-based methods for the same purpose is heavily restricted. In many cases, an inserted gene from a crossable species leads to the expression of a protein that is highly similar to those already expressed in the plant. Alternatively, additional copies of an endogenous gene may be introduced, resulting in elevated levels of the protein encoded by this gene. Also, if ZFN-3 is combined with cisgenesis to replace an endogenous sequence with a highly similar one from a cross-compatible species, it is likely challenging to develop suitable detection and quantification methods. In contrast, detection of the newly expressed protein is possible via protein-based methods if an inserted gene leads to the expression of a new protein previously not present in the plant.

At present, technically feasible methods such as computational subtraction (a method to filter away endogenous expected reads using reference sequences; Tengs et al., 2009) or fingerprinting by using a genome-walking type PCR to identify the plant genome sequence that flanks the transgenic/foreign sequence (Raymond et al., 2010) would be too costly and laborious for routine testing. Consequently, in the EU, GMO screening for enforcement purposes is currently mainly performed with methods for detecting DNA sequences and applying PCR technology (real-time PCR; EC, Joint Research Centre [JRC], Institute for Health and Consumer Protection (IHCP), 2011). They are mainly based on the detection of foreign DNA sequences like widely used promoters and terminators. As these by definition are absent in cisgenic plants, it is not possible to employ general screening methods. For cisgenic plants, information on the inserted genetic material and in particular the unique flanking sequences of the insertion site is necessary to develop an event-specific method for detection. Due to the currently available plant transformation methods, the integration occurs randomly and therefore results in a specific event with unique flanking sequences. The unequivocal detection and identification of a cisgenic modification is possible, and cisgenic organisms may be distinguished from their conventionally bred counterparts.

As stable integration—and thus changes in the genome—are not foreseen, detection of an agroinfiltrated plant will not be possible on the DNA level. It is generally presumed that the genome of an agroinfiltrated plant is free from any *Agrobacterium*-sequences and from vector sequences resulting from the transformation process itself. Intended solely for transient expression, no non-endogenous DNA-sequences are expected to be found in the host plant genome or in the progeny. Prior to further propagation of plant tissue infected with *Agrobacterium*, the plant material should be screened for the absence of any foreign DNA sequences (e.g., chromosomal *Agrobacterium* DNA or T-DNA). If free from foreign DNA, an organism resulting from agroinfiltration is not distinguishable from its conventional (wild type) counterparts on the DNA level.

Traceability

Traceability is largely based on documentation, and general traceability systems are foreseen in the major supply chains, food, feed and seed. Independent of the GMO status, the use of a new technique can easily be traced back by appropriate documentation if the applicant provides adequate information during the variety registration process. The presence of authorized GMOs is commonly traced by suitable detection methods, which are also necessary for surveillance purposes.

Specific legislative measures have been set into force concerning the traceability of GMOs; their applicability will depend on the classification (GM/non-GM status) of plant varieties developed through new breeding techniques.

If no authorization process according to GMO legislation is foreseen, the modified plants and derived products (food, feed, and seed) could not be detected in the control laboratory, and the only possibility to ensure traceability is by continuous documentation. The application of a new technique could be indicated in frame of the variety registration process, followed by continuous labeling or indication on accompanying papers. Within the established traceability systems, commodities and products may be traced back to the seed used for their production, and from the seed to the registered variety processed with new techniques.

Some genomic modifications are expected to be indistinguishable from those occurring during traditional plant breeding. For instance, point mutations induced by ODM or ZFN-1 techniques may be detected, but it is not possible to identify the origin of the mutation. In the past, numerous traditionally bred varieties

have been developed from plants mutated with radiation or chemicals, or based on spontaneously occurring, selected mutations. As the mutation may also be induced by the use of techniques like ODM or ZFN-1, its origin in a given plant may not be clarified. Consequently, information on the underlying technique has to be requested within the application procedure for variety registration if this is to be traced back.

Labeling

One key aspect for labeling of a GMO commodity or product is the availability of reliable protocols for unequivocal detection and quantification. Thresholds for the adventitious or technically unavoidable presence of authorized GMOs have been established, and the labeling provisions for products (material) are currently based on the unequivocal quantification of the presence of an authorized GMO.

Cisgenic plants harbor a unique combination of the inserted and its flanking sequences in the plant genome, which allows for event-specific, unequivocal detection and quantification. It can be difficult to quantify the presence of plant material developed through other new techniques, and thus to follow the labeling provisions according to the current GMO legislation. This is likely the case for ODM and ZFN-1 leading to point mutations, for which it is not easy to develop an unambiguous quantification method.

Risk Assessment

In transgenesis, DNA fragments from any organism and in any combination may be inserted into a genome, extending the gene pool of the recipient species and frequently creating completely artificial traits. Cisgenesis and intragenesis make use of genes from the same or cross-compatible species, which could therefore also be transferred by traditional breeding methods. In cisgenic plants, the gene of interest will be left contiguous and unmodified and must be inserted in sense orientation. However, additional sequences such as from the backbone of a plasmid may be introduced unintentionally. Intragenic plants in contrast may contain genetic elements that have been rearranged *in vitro*. Cisgenic and intragenic plants should be tested to confirm that they contain only the intended modifications and no foreign genes, which is in accordance with current European provisions (EC, 2013). Plants are, by definition, not cisgenic if foreign genes are present (Schouten et al., 2006). However, their present regulation as transgenic is under evaluation in a number of countries, including within the European Union.

Most of the risks associated with transgenic techniques are relevant for intragenic or cisgenic approaches as well, as in most cases the methods of choice used to deliver DNA into plant cells are the same as in transgenesis. These risks include unintended alterations to the genome beyond the insertion that may be caused by processes and mechanisms governing the generation of transgenic plants (European Food Safety Authority [EFSA], 2012). Cisgenic plants are usually produced through *Agrobacterium*-mediated transformation. There is scientific consensus that this method is prone to cause mutations like deletions and rearrangements within the plant genomic DNA (Schouten & Jacobsen, 2007; Wilson, Latham & Steinbrecher, 2006). *Agrobacterium*-mediated transformation causes the random insertion of the T-DNA and frequently leads to unintended insertions of additional sequences that are derived from the bacteria themselves, the vector backbone, or result from DNA rearrangements. In addition, deletions may occur. Following the definition of cisgenesis, the accurate elimination of selection marker sequences, if applicable, needs to be assessed. This refers to all inserted sequences that are not essential to achieve the desired trait, and in particular to antibiotic-resistance marker genes, which should be avoided (EC, 2013).

In any case, isolated genes and their regulatory elements are introduced into another position in the plant genome, different from the introgression of genes by traditional methods. As the donor sequence is inserted into the genome at an *a priori* unknown position, it might affect DNA methylation and other factors that in turn can influence gene expression. The insertion of a cisgene results in a mutation at the insertion site. Rearrangements or translocations might occur in the flanking regions. These mutations can knock out genes, open new reading frames, and thereby induce phenotypic effects. Thus, it has to be proven whether the cisgenes retain their anticipated function in an altered genetic background. In any case, it has to be taken into account that recombinant DNA technology is different from meiotic recombination, and unintended effects resulting from the transformation process have to be minimized to the extent possible.

Cisgenesis is defined as the insertion of a whole, unchanged gene of interest (including introns and regulatory elements) derived from a cross-compatible species. Intragenesis is not different from transgenesis except that all parts of the inserted DNA are derived from a cross-compatible species. Intramolecular recom-

bination (inversion, excision, deletion) prior to insertion is not limited by definition, and anti-sense orientation is possible.

The thorough molecular characterization is a crucial step in the risk assessment of plants resulting from the application of new techniques, as only a solid characterization of the DNA sequence of the insert and the flanking sequences can unequivocally prove their anticipated character. The molecular characterization in principle aims at the verification that indeed the desired modification has occurred in the plant genome and at the exclusion of unintended effects. In addition, it can provide insights into potential reasons for detrimental impacts resulting from the modification, such as the disruption of an endogenous gene at the insertion site or mutations in the genome different from the targeted one. Thus, for all plants produced through new techniques discussed here, the molecular characterization has to be as substantial as for transgenic plants. The molecular and phenotypic analyses should complement each other to provide a solid basis for further (case-by-case) decisions concerning the necessary elements in the risk assessment procedure.

The new techniques are largely equivalent to traditional breeding regarding the gene pool. As the gene has already been present in the cross-compatible population, a number of risks associated with the use of transgenic plants (e.g., plant-to-plant gene transfer) are presumably not relevant. Concerning food and feed safety assessment, reduced data requirements may be appropriate if the donor plant has been part of the diet, or the characteristics of the modified plant and derived products are not substantially different from what may be expected as a result of traditional methods. If the donor plant (and thus the introduced/mutated gene) has previously been part of the human diet, its safe use and consumption may be anticipated. Furthermore, it is reasonable to anticipate that the risks they pose for human and animal health are similar to those arising from the consumption of traditionally bred plants, and the food and feed safety assessment may be conducted accordingly. The safety of newly expressed proteins and metabolites, if applicable, has to be established.

The fact that a modified gene and, where appropriate, a newly expressed protein stem from the same or cross-compatible species or have been directly modified in the genome lowers the chances that potential adverse effects to the environment occur. If a gene has already been present in the (cross-compatible) population, a number of risks associated with transgenic plants are presumably not relevant.

When applying site-directed mutagenesis, no random integration, multiple insertions, or effects due to the insertion in undesirable locations in the genome may occur (Oh & May, 2001). The gene remains in its normal chromosomal context, thereby reducing the chances of altered gene expression, unless intended. In common applications, the endogenous expression patterns are expected to persist. Due to presumed target-site specificity and sequence dependency of the approach, it may be expected that the genomic environment is not markedly perturbed as with transgene integration (Zhu, Mettenburg, Peterson, Tagliani & Baszczynski, 2000). Targeted insertion of sequences generally also minimizes the chance of unintended alterations.

Despite their potential, scientific publications indicate that neither the efficiency nor the specificity of the technologies aiming at targeted alterations of plant genomes can be controlled sufficiently. Unintended effects cannot be excluded, e.g., off-target effects during ZFN-mediated gene modifications (Townsend et al., 2009).

In conclusion, the basis to assess potential risks arising from plants developed through new techniques and derived food and feed is similar to that applying to GM plants. Depending on the characteristics of the plant under investigation, the data requirements may be reconsidered case-by-case. In conclusion, it can be said that the current EU regulatory framework for genetically modified food and feed as well as the respective EFSA Guidance Document (EFSA, 2011) in general will be applicable also for plants resulting from the use of new plant-breeding techniques. However, it is possible that the application of new techniques results in plants not substantially different in their characteristics from those bred traditionally. Hence, elements of the risk-assessment procedure described in the respective EFSA Guidance Documents for the evaluation of food and feed products and for performing the environmental risk assessment, e.g. environmental risks caused by gene transfer, may be reconsidered. Food- and feed-safety aspects have to be generally taken into consideration for all newly developed plants.

Conclusions

Different scenarios are possible, which depend on regulatory decisions on the European level—to sustain the current risk assessment practices and leave crops produced through new techniques within the GMO regulatory framework, to adapt the current risk assessment for GMOs, or to exempt the crops under investigation from

the GMO regulatory requirements. These decisions will influence practical consequences concerning detection, traceability, labeling, and risk assessment.

As part of the EU's traceability and labeling requirements for post-marketing surveillance, site-specific sequence data for the entire inserted DNA, along with adjacent genomic sequences near the insertion site, are required for event-specific tracking purposes. Detection of plants and derived products developed by novel techniques (e.g., cisgenesis, intragenesis, ODM, ZFN, Agroinfiltration) is virtually impossible without information concerning the site of the genomic alteration. In case of plants subjected to site-directed mutagenesis, it is not possible to determine the origin of the mutation, rendering them indistinguishable from plants derived from other mutagenesis techniques, including naturally occurring ones. The availability of necessary information to allow detection and/or traceability should be assured by appropriate legislative measures, e.g., within the EU regulatory framework.

Considering the state of the art, plants derived by new plant-breeding techniques should be risk assessed based on the same principles and requirements (EU regulatory framework, respective EFSA Guidance Documents) as applied for transgenic plants. It has to be taken into account that recombinant DNA technology, even when employing sequences from the sexually compatible gene pool, is different from meiotic recombination. Unintended effects resulting from the transformation process or due to technical constraints have to be minimized to the extent possible but cannot be avoided sufficiently.

Based on comprehensive evaluation of the characteristics of the plant under investigation and corresponding information, the data requirements for regulators may be reduced on a case-by-case basis. General information on the technique employed during the breeding process would facilitate traceability. In this case, commodities and products may be traced back to the seed and further to a variety developed by using novel breeding techniques.

The classification of the resulting plants (GM/non-GM) is identified as crucial in determining the legislative measures that apply in terms of risk assessment, detection, and traceability, and derive practical consequences for regulators, stakeholders, and consumers.

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Acknowledgements

This study was supported in part by the Austrian Federal Ministry of Health.