Technical Summaries of New Plant Breeding Techniques-NBTs
June 2014

Breeders have long relied on plant breeding as a filter to eliminate undesirable characteristics in plants and develop plant varieties with desired characteristics that meet the needs of the breeder, farmer and consumer. Throughout history, as knowledge of plant biology and genome diversity advanced so did breeding tools, continuously resulting in new opportunities to enable rapid identification and breeding of desirable characteristics in plants. With today's understanding of biological variation and how it can be deployed to improve plant performance, knowledge-based tools have continued to advance allowing breeders to rapidly, reliably and predictably develop desirable characteristics in plants.

Several technical and regulatory organizations in various countries have recently been involved in assessing the need for regulatory oversight of plants resulting from a number of plant breeding techniques described as "New Plant Breeding Techniques" (NPBTs). This document provides a technical overview of some of these so-called NPBTs.

Oligonucleotide-Directed Mutagenesis (ODM)

Classical mutagenesis approaches (*e.g.* chemical/radiation) have been widely employed in the development of numerous commercial crops (*e.g.* see the FAO/IAEA mutant variety database at http://mvgs.iaea.org/AboutMutantVarities.aspx). Such approaches are based on the creation of a large pool of randomly mutagenized plants followed by years of breeding to filter out undesirable mutations and to identify plants with desired improved characteristics (Novak and Brunner, 1992). Over the past decade, a variety of knowledge-based mutational breeding tools, including ODM and Site Directed Nucleases (SDNs), have been developed that result in plant varieties with desirable characteristics in a more precise and efficient manner than classical breeding and mutagenesis approaches. Once developed, traditional breeding (filtering) processes are then deployed to efficiently breed the desirable trait into elite plant varities while eliminating undesirable characterizes, resulting in superior plant varities.

ODM is a tool for targeted mutagenesis, employing a specific oligonucleotide, typically 20-100 bp in length, to produce a single DNA base change in the plant genome (Beetham *et al.*, 1999; Zhu *et al.*, 1999).

The oligonucleotide is identical (homologous) to a unique, predetermined DNA sequence in the plant genome, with the exception of a single base-pair change. When cultured plant cells are temporarily exposed to these short oligonucleotide sequences (repair templates), the repair template binds to the corresponding homologous plant DNA sequence. Once bound, the cell's natural repair machinery recognizes the single base mismatch between its own DNA and that of the repair template. Due to this difference, the cell will repair its DNA sequence by copying the mismatch into its own DNA sequence. As a result, the desired specific change in the plant's genome is produced and the oligonucleotide is subsequently degraded by the cell. Plants carrying the specific mutation are subsequently regenerated by tissue culture techniques and traditional breeding is deployed to efficiently breed the desirable trait into elite plant varities while eliminating undesirable characterizes.

ODM does not lead to the insertion of foreign DNA into the plant genome and the final product produced is similar and indistinguishable from plants that could arise though spontaneous mutations or by application of classical mutagens. The ODM technique has been successfully applied in several plant crops, *e.g.* to generate herbicide tolerance (Beetham *et al.*, 1999; Zhu *et al.*, 1999; Okuzaki and Toriyama, 2004; Dong *et al.*, 2006). In addition, ODM has the potential to take advantage of the plant's own genome and improve crops through enhanced disease resistance (insect, bacterial, virus), improved nutritional value and enhanced yield without the introduction of new genetic material.

Beetham, P.R., Kipp, P.B., Sawycky, X.L., Arntzen, C.J., May, G.D., 1999. A tool for functional plant genomics: Chimeric RNA/DNA oligonucleotides cause *in vivo* gene-specific mutations. PNA 96, 8774-8778.

Dong, C., Beetham, P.R., Vincent, K., Sharp, P., 2006. Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. Plant Cell Reports 25, 457-465.

Novak, F.J., Brunner, H., 1992. Plant breeding: Induced mutation technology for crop improvement. IAEA Bulletin, 25-33.

Okuzaki, A., Toriyama, K., 2004. Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. Plant Cell Report 22, 509-512.

Zhu, T., Peterson, D.J., Tagliani, L., St. Clair, G., Baszczynski, C.L., Bowen, B., 1999. Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. PNAS 96, 8768-8773.

Site-directed Nucleases (SDN)

SDN–based technologies allow for knowledge-based targeted genome improvements in plants, including single nucleotide changes, DNA deletions or insertions. The technology relies on custom-designed DNA nucleases to introduce a double-stranded break (DSB) at a predetermined, specific genomic location. Repair of this DSB by the cell's natural repair process leads to the introduction of mutations or DNA insertions at the break site (Tzfira *et al.*, 2012).

SDN-based technologies encompass a range of applications that were first, and continue to be, successfully used in animal and human cells, and are now being developed for use in plants (Tzfira *et al.*, 2012; Podevin *et al.*, 2013). Several different types of SDNs are currently being utilized in plants, including Meganucleases (MN), Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated proteins (Xie and Yang, 2013).

In each case, SDNs consist of a DNA binding region (protein, or in the case of CRISPRs, RNA) and an endonuclease (*e.g. Fok*I, Cas9). The DNA binding regions are custom-designed to bind to a unique, predetermined DNA sequence in the plant genome. Once bound to the unique DNA sequence, the endonuclease results in a targeted DSB in the plant DNA. This process activates the natural cell repair mechanisms to repair the DNA break, creating small deletions, nucleotide substitutions or DNA additions at the site of the targeted DSB.

Variants of SDN applications are often categorized as SDN-1, SDN-2 and SDN-3 depending on whether the nuclease is used alone or in combination with a DNA-repair template.

SDN-1: If the SDN is delivered alone (SDN-1), the targeted DSB in the plant genome is repaired through the predominant Non-Homologous End Joining (NHEJ) repair system in plants, an error prone process that rejoins DNA ends together and may result in small, nucleotide deletions, additions or substitutions. Alternatively, if two DSBs are induced on either side of a target DNA sequence, SDN-1 can result in removal of larger DNA regions (*e.g.* promoter or whole gene).

SDN-2: Another variation of the SDN technique (SDN-2) uses an SDN for the targeted DSB along with a DNA repair template. The repair template is identical to the DSB target site except for one or a few nucleotide changes. The cell utilizes the homologous template to repair the DNA break by homology-directed repair, thereby incorporating the small nucleotide changes (point mutations of one or a few base pairs) into the targeted DSB.

SDN-3: In the third variant of SDN (SDN-3), just like in SDN-2 a DNA repair template is delivered into the cell to repair the DSB. However, in the case of SDN-3, the repair template mismatch is much larger, for example, a gene expression cassette, resulting in targeted gene addition.

SDNs may be delivered to the cells in various ways; examples of successful delivery methods include direct delivery of DNA or RNA (protoplast transfection), virus-aided gene expression through infection by a non-integrating RNA virus encoding SDN, or transient transformation of explants with a DNA sequence encoding the SDN (without transgene integration). Alternatively, SDNs may be stably integrated in a donor intermediate plant line and transiently crossed into a recipient plant line for targeted genomic improvements. After the cross, the SDN is expressed and then segregated away from the recipient line resulting in a final product containing only the targeted modification.

The advantage of SDN technologies compared to classical mutagenesis is the precision with which such variation can be created and the ability to rapidly screen and identify the desired product. Once the desired product is identified, traditional breeding is deployed to efficiently breed the desired trait into elite plant varieties while eliminating undesired characterizes.

As SDN technologies continue to advance, the existing suite of SDNs is expected to expand further to include other customized enzymes for targeted genome engineering (Gaj *et al.*, 2013). Whatever the type of SDN and delivery method employed, these precision breeding approaches allow for the development and the capture of local genetic variation in plants and result in a final product that may be similar or indistinguishable from products that could be obtained through conventional plant breeding or mutagenesis techniques.

Gaj, T., Gersbach, C.A., Barbas III, C.F., 2013. Zfn, talen, and crispr/cas-based methods for genome engineering. Trends in Biotechnology 31, 397-405.

Podevin, N., Davies, H.V., Hartung, F., Nogue´, F., Casacuberta, J.M., 2013. Site-directed nucleases: A paradigm shift in predictable, knowledge-based plant breeding. Trends in Biotechnology 31, 375-383.

Tzfira, T., Weinthal, D., Marton, I., Zeevi, V., Zuker, A., Vainstein, A., 2012. Genome modifications in plant cells by custom-made restriction enzymes. Plant Biotechnology Journal 10, 373-389.

Xie, K., Yang, Y., 2013. RNA-guided genome editing in plants using a crispr-cas system. Molecular Plant 6, 1975-1983.

Cisgenesis and Intragenesis

Cisgenesis and intragenesis refer to the introduction of DNA into a plant that is derived from the same or a sexually compatible species. The approach allows for useful genes from the same species to be efficiently transferred to elite cultivars and overcome limitations of classical breeding such as linkage drag and lengthy backcrossing; other uses are in plants which are difficult to sexually cross, e.g. potato, or those with long generation cycles, such as fruit or woody trees (Holme *et al.*, 2013).

Whereas cisgenesis refers to the introduction of whole genes (with their own promoter, coding sequence including exons and introns, and terminator), a variation of this approach, called intragenesis, employs chimeric genes containing specific elements taken from different genes within the same (sexually compatible) species, *e.g.* a different promoter to enhance expression of the target gene or an inverted sequence to silence expression of an endogenous gene. Cisgenic/intragenic DNA is free of foreign DNA, but, in the case of *Agrobacterium*-mediated transformation, may contain short stretches of T-DNA border sequences that are non-coding, have no function in the plant, and may occur already elsewhere in the genome.

Cisgene or intragene insertion may be accomplished by either recombinant DNA techniques or by site-directed nuclease technology, whereby the desired gene copy is part of the DNA repair template for homologous recombination at the targeted site (SDN-3).

Cisgenesis and intragenesis result in DNA duplication or insertion of a known wild-type version of a gene into an elite germplasm. Gene duplication plays a central role in plant diversification and evolution and is the basis for the occurrence of multigene families and clusters of *e.g.* disease resistance genes (Flagel and Wendel, 2009). Sequence analysis of current plant genomes has revealed the occurrence of many duplicated gene copies and copy number variations between genotypes are common (Springer *et al.*, 2009). The soybean genome *e.g.* already contains multiple copies of 75% of all its genes (Schmutz *et al.*, 2010).

In light of the highly dispersed plant genome, addition of a new copy of a gene that already exists within the species (*e.g.*, a cisgene) is not different from the natural processes typical of plant genomes (ACRE, 2013) and can be considered a specific form of (partial) self-cloning, a phenomenon that is also widespread in bacteria. At the genome level, intragenesis may be considered to be similar to natural processes acting on plant genomes. *E.g.* 85% of the maize genome consists of transposable elements (Schnable *et al.*, 2009), some of which are known to capture pieces of DNA from throughout the genome and assemble them into novel combinations; these processes have creating new chimeric proteins that may have contributed to many of the genotype-specific differences occurring in plants (Weber *et al.*, 2012).

Cisgenic and intragenic plants express the same proteins that already occur in the species, but at a modified level or in a different pattern, and are therefore comparable to plants developed by conventional breeding or natural evolution.

ACRE, 2013. Acre advice: New techniques used in plant breeding.

Flagel, L.E., Wendel, J.F., 2009. Gene duplication and evolutionary novelty in plants. New Phytologist 183, 557-564.

Holme, I.B., Wendt, T., Holm, P.B., 2013. Intragenesis and cisgenesis as alternatives to transgenic crop development. Plant Biotechnology Journal 11, 395-407.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.-C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., Jackson, S.A., 2010. Genome sequence of the palaeopolyploid soybean. Nature 463, 178-183.

Schnable, P.S., Ware, D., Fulton, R.S., Stein, J.C., Wei, F., Pasternak, S., Liang, C., Zhang, J., Fulton, L., Graves, T.A., Minx, P., Reily, A.D., Courtney, L., Kruchowski, S.S., Tomlinson, C., Strong, C., Delehaunty, K., Fronick, C., Courtney, B., Rock, S.M., Belter, E., Du, F., Kim, K., Abbott, R.M., Cotton, M., Levy, A., Marchetto, P., Ochoa, K., Jackson, S.M., Gillam, B., Chen, W., Yan, L., Higginbotham, J., Cardenas, M., Waligorski, J., Applebaum, E., Phelps, L., Falcone, J., Kanchi, K., Thane, T., Scimone, A., Thane, N., Henke, J., Wang, T., Ruppert, J., Shah, N., Rotter, K., Hodges, J., Ingenthron, E., Cordes, M., Kohlberg, S., Sgro, J., Delgado, B., Mead, K., Chinwalla, A., Leonard, S., Crouse, K., Collura, K., Kudrna, D., Currie, J., He, R., Angelova, A., Rajasekar, S., Mueller, T., Lomeli, R., Scara, G., Ko, A., Delaney, K., Wissotski, M., Lopez, G., Campos, D., Braidotti, M., Ashley, E., Golser, W., Kim, H., Lee, S., Lin, J., Dujmic, Z., Kim, W., Talag, J., Zuccolo, A., Fan, C., Sebastian, A., Kramer, M., Spiegel, L., Nascimento, L., Zutavern, T., Miller, B., Ambroise, C., Muller, S., Faga, B., Yeh, C.-T., Barbazuk, W.B., Chaparro, C., Jeddeloh, J.A., Spooner, W., Levy, M.J., Emrich, S.J., Baucom, R.S., Chia, J.-M., Han, Y., Narechania, A., McMahan, L., Jia, Y., Brutnell, T.P., Deragon, J.-M., Ren, L., Buren, P.V., Kalyanaraman, A., Carpita, N.C., Estill, J.C., Lee, H., Wei, S., Vaughn, M.W., Hsia, A.-P., Li, P., Kumari, S., Ying, K., Fu, Y., Lisch, D.R., Liu, S., Myers, A.M., Schneider, K.L., Springer, N.M., Wolfgruber, T.K., Bennetzen, J.L., Wessler, S.R., McCombie, W.R., Liu, Z., Nettleton, D., Schwartz, D.C., Sun, Q., Yang, L., Dawe, R.K., Wing, R.A., Nagel, D.H., Nguyen, J., Sharma, A., Wang, H., Yu, Y., Aluru, S., Wilson, R.K., Penning, B.W., Soderlund, C., Zhang, L., Jiang, J., McCann, M.C., Waterman, M., Zhou, S., Jiang, N., SanMiguel, P., Ponnala, L., Westerman, R., Zhu, Q., Presting, G.G., Martienssen, R.A., Clifton, S.W., 2009. The b73 maize genome: Complexity, diversity, and dynamics. Science 326.

Springer, N.M., Ying, K., Fu, Y., Ji, T., Yeh, C.-T., Jia, Y., Wu, W., Richmond, T., Kitzman, J., Rosenbaum, H., Iniguez, A.L., Barbazuk, W.B., Jeddeloh, J.A., Nettleton, D., Schnable, P.S., 2009. Maize inbreds exhibit high levels of copy number variation (cnv) and presence/absence variation (pav) in genome content. PLoS Genetics 5, e1000734.

Weber, N., Halpin, C., Hannah, L.C., Jez, J.M., Kough, J., Parrott, W., 2012. Crop genome plasticity and its relevance to food and feed safety of genetically engineered breeding stacks. Plant Physiology 160, 1842-1853.

Reverse Breeding

Hybrid breeding is an established breeding process in many commercial crops (*e.g.* maize) whereby elite homozygous inbred plant lines are crossed to yield an F1 hybrid (heterozygous) variety with superior performance due to heterosis (hybrid vigor) effects. In many crops, large collections of homozygous breeding lines are lacking, and other approaches are needed for the propagation of superior heterozygous plants. This is made possible by reverse breeding, a process that produces homozygous plants from a heterozygous superior plant to be used for reconstruction of a superior F1 genotype (Dirks *et al.*, 2009).

To perform reverse breeding, the original elite plant is first genetically transformed with a construct suppressing meiotic recombination through silencing of endogenous genes involved in the meiotic recombination process; microspores that are subsequently formed on this plant are induced to develop into new plants by double haploid induction techniques. Following screening of the resulting plants with genetic markers, doubled haploids are selected that, when crossed, would contain all genetic information from the original heterozygous line, and which are free of transgene DNA.

The selected double haploids can be used as inbred lines in hybrid breeding programs. The resulting F1 hybrid is genetically and phenotypically similar to the original superior plant and does not contain any foreign DNA. Moreover, variation in meiotic recombination is a natural occurring phenomenon (Wijnker and De Jong, 2008).

An alternative approach for silencing of meiotic recombination genes involves use of virus-induced gene silencing (see Section 4); in this case, no inducer plant is used in the reverse breeding process (Dirks *et al.*, 2009). Reverse breeding has had only limited success up to now and may have use in specialty crops only.

Dirks, R., van Dun, K., de Snoo, C.B., van den Berg, M., Lelivelt, C.L.C., Voermans, W., Woudenberg, L., de Wit, J.P.C., Reinink, K., Schut, J.W., van der Zeeuw, E., Vogelaar, A., Freymark, G., Gutteling, E.W., Keppel, M.N., van Drongelen, P., Kieny, M., Ellul, P., Touraev, A., Ma, H., de Jong, H., Wijnker, E., 2009. Reverse breeding: A novel breeding approach based on engineered meiosis. Plant Biotechnology Journal 7, 837-845.

Wijnker, E., De Jong, H., 2008. Managing meiotic recombination in plant breeding. Trends in Plant Science 13, 640-646.

RNA-dependent DNA methylation (RdDM)

Targeted Alteration of Gene Expression Through Epigenetic Control

Epigenetic variation refers to heritable changes in gene expression that are not caused by changes in the DNA sequence. Such variation is a widespread mechanism in plants and other organisms to regulate gene expression in response to developmental or environmental changes.

In plants, such changes often result from DNA methylation of specific genes and the phenomenon is mediated by small interfering RNA (siRNA), hence the name RNA-dependent DNA methylation (RdDM) (Mathieu and Bender, 2004; Daxinger *et al.*, 2009; Becker *et al.*, 2011). Epigenetic effects often work as a kind of biological memory, induced by environmental or other stimuli: induction of an epigenetic phenotype, *e.g.* drought resistance, in a parent line by exposure to drought conditions may result in a similar phenotype in the progeny, even without prior exposure to drought. Such epigenetic changes may persist over one or a number of generations, but the effect gradually fades away due to the natural removal of the methyl groups.

Specific epigenetic effects can be induced by insertion of a transgenic RNAi construct in a parental plant to silence expression of a target gene, then segregating the transgenic RNAi construct away in the next generation. The resulting plant may still reveal the epigenetic effect without containing the RNAi construct. Alternatively, the epigenetic effect may result from virus-induced gene silencing following inoculation with an engineered plant RNA or DNA virus containing a fragment of the target gene, without integration of any foreign DNA (Kanazawa *et al.*, 2011).

Epigenetic effects that occur without any genetic changes to the plant DNA are comparable to natural epigenetic regulation of gene expression (Becker *et al.*, 2011; Holeski *et al.*, 2012). Breeding for specific epigenetic effects and the selection of desirable offspring, regardless of the employment of an intermediate inducer plant, is not different from conventional breeding.

Becker, C., Hagmann, J., Müller, J., Koenig, D., Stegle, O., Borgwardt, K., Weigel, D., 2011. Spontaneous epigenetic variation in the *Arabidopsis thaliana* methylome. Nature 480, 245-249.

Daxinger, L., Kanno, T., Bucher, E., van der Winden, J., Naumann, U., Matzke, A.J.M., Matzke, M., 2009. A stepwise pathway for biogenesis of 24-nt secondary sirnas and spreading of DNA methylation. The EMBO Journal 28, 48-57.

Holeski, L.M., Jander, G., Agrawal, A.A., 2012. Transgenerational defense induction and epigenetic inheritance in plants. Trends in Ecology & Evolution 27, 618-626.

Kanazawa, A., Inaba, J.-i., Kasai, M., Shimura, H., Masuta, C., 2011. RNA-mediated epigenetic modifications of an endogenous gene targeted by a viral vector—a potent gene silencing system to produce a plant that does not carry a transgene but has altered traits. Plant Signaling & Behavior 6, 1090-1093.

Mathieu, O., Bender, J., 2004. RNA-directed DNA methylation. Journal of Cell Science 117, 4881-4888.

Virus-Induced Transient Gene Expression or Gene Silencing

Modified plant viruses have been used for many years for transient silencing of plant genes to study plant gene function (Lu *et al.*, 2003; Zhang *et al.*, 2010; Lange *et al.*, 2013). More recent research has shown the induction of early flowering following infection of apple tissues with apple latent spherical virus (ALSV) vector, an RNA virus, expressing a flowering time gene and/or silencing a terminal flower gene (Yamagishi *et al.*, 2011; Yamagishi *et al.*, 2013). In this case, apple seed embryos were inoculated with ALSV vector RNA, which is translated inside the plant cells into protein and/or replicated and metabolized to siRNA which triggers target gene silencing. The RNA virus did not integrate into the plant genome, was not transmitted to progeny, nor spread horizontally between plants.

Such applications may be particularly useful to reduce breeding times by several years in (fruit) trees; the process would include inducing early flowering in one parental tree, then use pollen from that tree to pollinate a traditional tree to produce offspring for further breeding. Plants resulting from this process reveal no changes to their genome and are indistinguishable from other plants produced by breeding. The virus-induced gene silencing process mimics widespread plant defense systems against viral infection.

Lange, M., Yellina, A.L., Orashakova, S., Becker, A., 2013. Virus-induced gene silencing (vigs) in plants: An overview of target species and the virus-derived vector systems. Virus-Induced Gene Silencing: Methods in Molecular Biology 975, 1-14.

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., Baulcombe, D., 2003. Virus-induced gene silencing in plants. Methods 30, 296-303.

Yamagishi, N., Kishigami, R., Yoshikawa, N., 2013. Reduced generation time of apple seedlings to within a year by means of a plant virus vector: A new plant-breeding technique with no transmission of genetic modification to the next generation. Plant Biotechnology Journal doi: 10.1111/pbi.12116, 9 pages.

Yamagishi, N., Sasaki, S., Yamagata, K., Komori, S., Nagase, M., Wada, M., Yamamoto, T., Yoshikawa, N., 2011. Promotion of flowering and reduction of a generation time in apple seedlings by ectopical expression of the *Arabidopsis thaliana* ft gene using the *apple latent spherical virus* vector. Plant Molecular Biology 75, 193-204.

Zhang, C., Bradshaw, J.D., Whitham, S.A., Hill, J.H., 2010. The development of an efficient multipurpose bean pod mottle virus viral vector set for foreign gene expression and RNA silencing. Plant Physiology 153, 52-65.

Accelerated Plant Breeding Employing a GM Inducer Plant

Breeding times in plants with long juvenile phases can be significantly shortened by induction of early flowering in parental generation(s), then segregating the transgenes away in subsequent breeding cycles. Such early flowering plants could dramatically accelerate breeding of forest (poplar, Eucalypt, etc.) and fruit trees.

Early flowering genotypes have been obtained in apple with an ALVS vector, but also by stable insertion of foreign flowering genes in an inducer plant (reviewed in (Flachowsky *et al.*, 2009). Such early flowering inducer plants may flower within a year (compared to 5-10 years) and can be crossed with plants during one or more generations, allowing *e.g.* to introgress disease resistance genes from wild apple into elite apple cultivars in a high-speed breeding program (Flachowsky *et al.*, 2011).

At a later breeding cycle, progeny is selected that lacks the transgene insertion and the associated early flowering phenotype. Selected progeny that does not contain the transgenes is genetically invariable compared to plants resulting from conventional breeding and detection of such plants is therefore impossible.

Flachowsky, H., Hanke, M.-V., Peil, A., Strauss, S.H., Fladung, M., 2009. A review on transgenic approaches to accelerate breeding of woody plants. Plant Breeding 128, 217-226.

Flachowsky, H., Le Roux, P.-M., Peil, A., Patocchi, A., Richter, K., Hanke, M.-V., 2011. Application of a high-speed breeding technology to apple (*malus × domestica)* based on transgenic early flowering plants and marker-assisted selection. New Phytologist 192, 364-377.

Agroinfiltration

Agroinfiltration is not a new technique in plant breeding and consists of local infiltration or inoculation of plant tissues (usually leaves) with *Agrobacterium* cells to deliver a T-DNA vector containing a gene expression cassette. The response of the plant to the expression of the introduced proteins or silencing factors (RNAi) is monitored to select plants for further breeding. The technique is mainly applied as a diagnostic tool for disease resistance testing in some crops. As the *Agrobacterium* cells are applied locally to vegetative tissues, the T-DNA is not stably integrated in the germline[1] and is therefore not transmitted to the progeny.

Grafting onto a GM Rootstock

Grafting is an agrotechnical practice used by growers for thousands of years to control growth and flowering in trees and some herbaceous plants by attaching a scion (the upper part of the plant without the roots) onto a suitable rootstock (examples include fruit trees, grapes, tomatoes, cucumber, roses, *etc.*).

GM rootstocks have been developed that *e.g.* combat certain soil-borne diseases in fruit trees and grapes, and allows use of the same rootstock to support growth of many different varieties used as non-GM scion. Although the rootstock is GM, the variety grafted onto this rootstock as a scion is not genetically modified, nor are the fruits or seeds, and these are indistinguishable from conventional fruits or seeds.

Although proteins and RNA have been reported to be transported to the scion through a graft, this seems not to occur consistently (Flachowsky *et al.*, 2012), is not specific to the use of GM rootstocks, and the effects would be limited and are not heritable.

Flachowsky, H., Tränkner, C., Szankowski, I., Waidmann, S., Hanke, M.-V., Treutter, D., Fischer, T.C., 2012. RNA-mediated gene silencing signals are not graft transmissable from the rootstock to the scion in greenhouse-grown apple plants *malus* sp. International Journal of Molecular Sciences 13, 9992-10009.

[1] A variation of this technique, applied to inflorescences (called floral dip), is used for stable transformation mostly in *e.g. Arabidopsis*.