




Opportunities of New Plant Breeding Techniques



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Prospects of New Plant Breeding Techniques in crops

Plant breeding has resulted in numerous high-quality food, feed, ornamental and industrial crop varieties that are cultivated nowadays. Although traditional breeding based on the steps of crossing and selection remains an ongoing activity for crop improvement, it faces limitations as many crops have complex genetics (e.g. because not all crosses produce fertile offspring) or a long generation time (e.g. fruit trees).

Since the turn of the century several new tools and techniques have been invented or conceived and are being implemented to facilitate breeding of new improved crop varieties. Compared to traditional breeding these techniques reduce the time and effort needed to produce new crop varieties. These techniques are referred to as 'new plant breeding techniques'. Several of these new plant breeding techniques result in improved plants that can be obtained with traditional breeding as well (although through a very time consuming process). Table 1 summarizes the classification of the improved plants obtained with the different new plant breeding techniques.

Table 1 Classification of final products from new plant breeding techniques.

Technique	What is the final product after breeding?		
	Improved plant 1: Plant with new genes at new chromosomal locus	Improved plant 2: Plant without new genes but with a mutation	Improved plant 3: Plant without new genes or modifications
Cisgenesis	X ¹		
Intragenesis	X ¹		
Sequence-specific nuclease technology (SSN-1, SSN-2)		X	
Sequence-specific nuclease technology (SSN-3)	X ²	X ³	X ⁴
Oligo-directed mutagenesis (ODM)		X	
RNA-dependent DNA methylation			X
Reverse breeding			X
Induced early flowering			X
Grafting on GM rootstock			X

¹⁾ new DNA is originating from the same or closely related species,

²⁾ for targeted integration of cisgenes or intragenes at a specified location,

³⁾ for gene replacement with a modified (artificially changed) allele (modified cisgene),

⁴⁾ for gene replacement with a natural allele (cisgene)

A common denominator of most of the new plant breeding techniques is that they involve steps in which at some point DNA is directly introduced into the plant. This DNA can be in the form of an extra copy of natural variants of important genes (Fig. 1, Improved Plant 1). Alternatively, recombinant DNA is introduced temporarily and induces a mutation in a gene that is already present in the plant's genome, or delete the gene or its regulatory sequences (Fig. 1, Improved Plant 2). In a third variant of new plant breeding techniques, temporarily introduced recombinant DNA changes the expression of one or more genes that are present in the plant, but does not cause any change to the DNA itself (Fig. 1, Improved Plant 3).

When an inserted gene is present in a new variety (Improved Plant 1 in Fig. 1) the EC directive 2001/18/EC on Genetic Modification (GM) applies, but discussions are ongoing about how stringently these plants would have to be evaluated for safety, or whether they can be deregulated. For plants produced by new plant breeding techniques which do not contain new DNA fragments (Improved Plant 2 and 3 in Fig.1) the status under EU regulations is unclear.

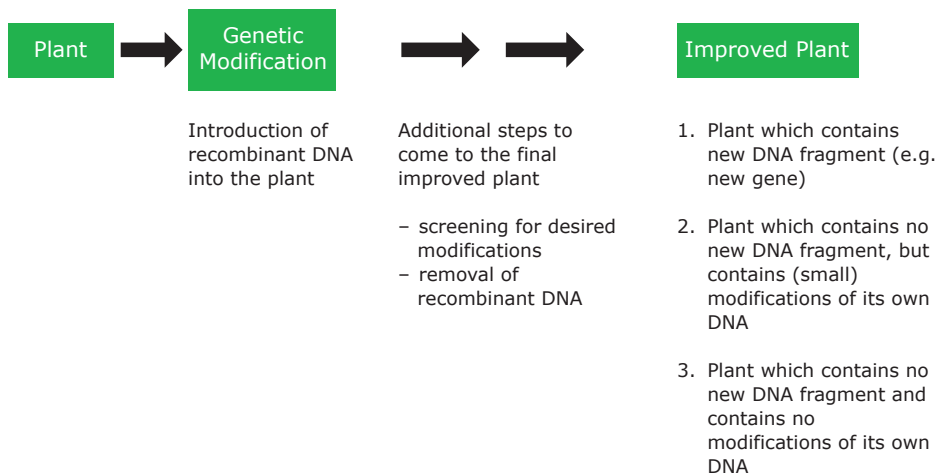


Figure 1 Outline of the process followed by new plant breeding techniques. In all new plant breeding techniques the process of producing an improved plant starts with the introduction of a recombinant DNA (or, in some variants, RNA) into the plant. Through subsequent selection and breeding steps the final products are produced. These can be classified into three classes with a different degree of genetic modification.

The aim of this brochure is to give an overview of new plant breeding techniques. This overview is based on a more technical review of the scientific literature, published in a separate report¹. The overview presents the opportunities and limitations of these techniques from the point of view of potential applications in plant breeding with promising results for improving agricultural sustainability. As the techniques have a rather different approach, we will first briefly present and describe them.

New plant breeding techniques reduce the time and effort needed to produce new varieties.

New plant breeding techniques make use of steps that qualify as genetic modification as at some point in time (recombinant) DNA is introduced into the plant.

In many cases, the resulting new varieties are essentially not different from what could be obtained by traditional methods.

Three types of end products can be distinguished:

- 1 Plants containing new DNA fragments
- 2 Plants with mutations in their DNA
- 3 Plants without new DNA fragments and without mutations.

¹ Schaart, J.G., van de Wiel, C.C.M., Lotz, L.A.P., Smulders, M.J.M. (2015) Opportunities for products of new plant breeding techniques. Submitted for publication.

Overview of New Plant Breeding Techniques

We briefly describe the new plant breeding techniques as they are listed also by the 'New Techniques Working Group of the European Commission'. Technically speaking, the first two (cisgenesis and intragenesis; Table 1) concern the origin of the genes used for the genetic modification, rather than a specific technology used to modify the DNA, and hence there is some overlap between the techniques.

Cisgenesis

Cisgenesis is the production of plants by genetic modification using only genes from the species itself or from a species that can be crossed with this species using traditional methods. The genes used are added as an extra copy and are natural variants with improved characteristics, for example giving resistance to a disease. In contrast, in transgenesis the genes inserted are from species outside the species' gene pool. A cisgenic plant can in principle also be produced through traditional breeding, but this would require a much longer period of time. Cisgenesis is a promising tool for the further improvement of elite crop varieties, especially in crops that are vegetatively propagated to maintain their genetic composition, such as apple, grape, potato, cassava and banana. The unique insertion site of the gene in cisgenic plants can be detected by molecular genetic methods. From a technical point of view, the application of cisgenesis for the introduction of disease resistance genes in potato and apple can soon reach the level of commercial application.

Cisgenic plants contain new genes or gene copies that originate from the species itself or from a species that can be crossed with the species using traditional methods (i.e. the species' normal gene pool). Cisgenic plants can be detected by molecular genetic methods.

Intragenesis

Intragenesis is similar to cisgenesis, as all elements that are introduced by genetic modification are present within the species or in a crossable species. The difference is that they are introduced as a new combination of functional elements of different genes (Fig. 2). Making such new combinations creates new possibilities, e.g. regarding when and where in the plant a gene is activated. This can for example be used by combining a gene with a stronger promoter to enhance the expression of resistance or pigmentation genes. This will result in higher levels of resistance or more intense pigmentation. The result of intragenesis cannot be achieved through normal breeding as the new

combinations are unlikely to arise in the breeding process. The combinatory nature of the introduced intragene as well as the insertion site in intragenic plants can be detected by molecular genetic methods.

Intragenic plants contain new genes or gene copies that originate from the species itself or from a species that can be crossed with the species using traditional methods. The genes are introduced as a new combination of genetic elements that cannot be obtained with traditional breeding. Intragenic plants can be detected by molecular genetic methods.

Sequence-specific nuclease technology

Sequence-specific nucleases (SSN) are synthetic proteins that are designed in the lab. These proteins bind to a specific DNA target sequence and induce a lesion at that position in the DNA. With this technology genes at any location in the DNA can be stably knocked-out, mutated, or replaced. The DNA lesion will be repaired by the plants' native DNA repair machinery. As repair of DNA breaks is relatively inaccurate, plants may be recovered that have a small deletion in the repaired DNA that disrupts the function of the gene.

The application of using SSN followed by selection of plants in which the lesion was inaccurately repaired is referred to as SSN-1 (Fig. 3). The native DNA repair machinery can also use a copy of the gene as a template for accurate repair. If along with the SSN construct also a modified copy of the original DNA sequence (with desired mutations) is provided, plants can be recovered in which these modifications are copied into the DNA. In this way mutations can be induced at an exact position, for instance to repair a defective gene (as in gene therapy) or to knock out an intact gene. This way of using SSN together with a small DNA repair template is referred to as SSN-2 (Fig. 3).

If the repair template contains a large extra fragment (for example a complete gene) instead of a few modifications, plants can be recovered in which this large fragment is incorporated at the location of the DNA lesion. This way of using SSNs is intended to introduce new genes (cisgenes, intragenes or transgenes) at a predefined position in the plant genome and is referred to as SSN-3 (Fig. 3). After the intended SSN-induced modification is achieved, the recombinant genes that code for the SSN proteins can be removed from the plant again.

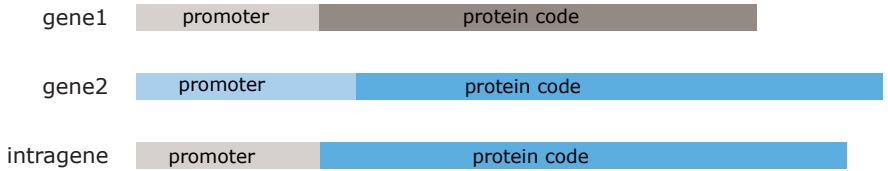


Figure 2 Intragenesis makes use of new combinations of functional elements of different natural genes. In the intragene shown the promoter of gene 1 is combined with the protein coding DNA sequence of gene 2. The gene promoter contains information for the specific regulation of the activity of the gene.

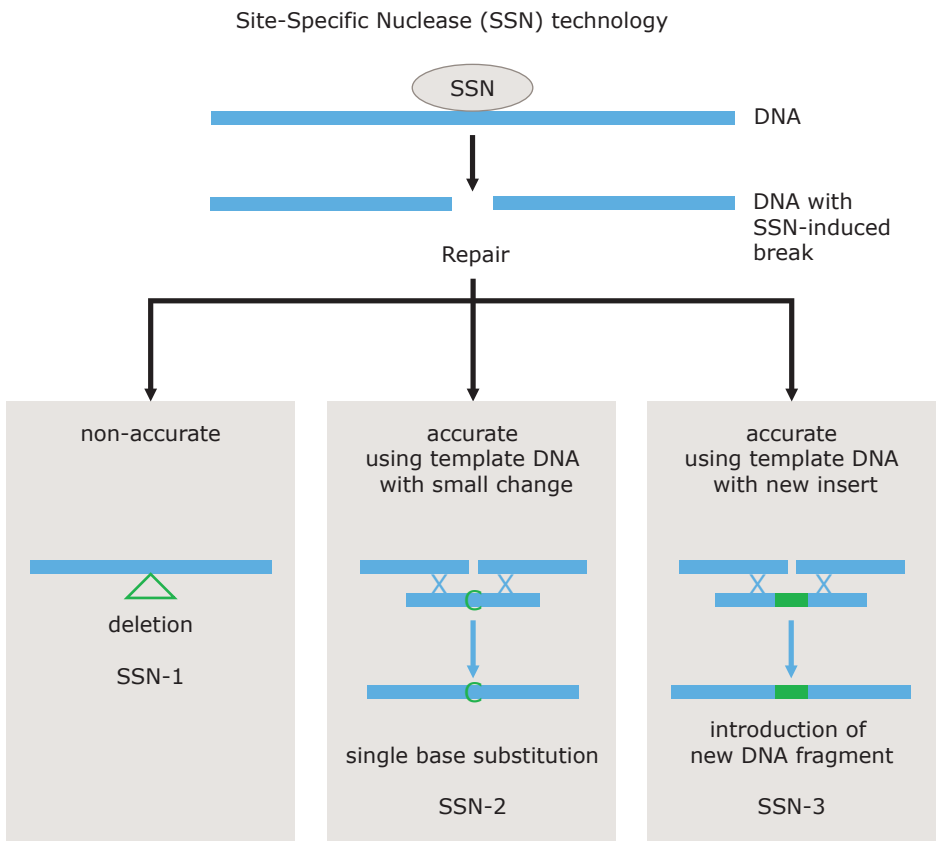


Figure 3 Outline of Sequence-Specific Nuclease technology SSN-1, SSN-2 and SSN-3. Note that the SSN is engineered such as to cause a break at an exact predefined location in the DNA.

Mutations produced using SSN-1 or SSN-2 may also be obtained using traditional mutagenesis methods (using mutagenic chemicals or ionizing radiation). These traditional mutagenesis methods however, produce random mutations throughout the genome and require therefore not only a selection step to find the intended mutations, but also several subsequent generations of backcrossing with elite plants to remove most of the undesired mutations.

On the technical side, Zinc Finger Nucleases (ZFNs), Meganucleases, TALENs and CRISPR-Cas9 are different variants of SSN. These can all be employed for the different SSN-1, -2, -3 applications. CRISPR-Cas9 is the latest and currently most promising SSN-tool. The application of SSN technology for the deletion of specific gene functions in crops will soon come to a point leading to commercial application.

Sequence-specific nuclease (SSN) technology

Using SSN technology any gene of interest can be stably knocked out, mutated, or replaced. SSNs bind to a specific target DNA sequence and induce a break in the DNA (a 'lesion'), which will be often inaccurately repaired by the plants' own repair mechanism, or which will induce accurate repair using a DNA-repair template.

Three types of SSN-applications can be distinguished:

- **SSN-1:** Gene knock-out. Inaccurate repair of the lesion results in deletions in the DNA, causing loss of gene function.
- **SSN-2:** Targeted mutation. If also a copy of the target DNA region is introduced that contains a small modification, the plant will use this as template for the repair of the lesion, and the modification will be copied into the gene as a mutation.
- **SSN-3:** Gene replacement. If the repair template contains a complete new gene, this will be inserted at the location of the lesion. Such genes can be cisgenes, intragenes or transgenes.

Mutations produced with SSN-1 and SSN-2 can be also obtained using traditional mutagenesis. The benefit of SSN technology over traditional mutagenesis is that it produces only a mutation at the desired location. In traditional mutagenesis thousands of mutations are produced in many plants, which makes it difficult to find the desired mutation. Moreover, it is time-consuming to remove the unwanted mutations. ZFN, TALENs and CRISPR-Cas9 are different examples of SSN.

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM) is partly similar to SSN-2 as also here small repair templates are introduced in plant cells, and plants are selected in which a modification in the template is copied into the DNA. The difference is that no gene construct is introduced into the plant to induce a DNA break. Instead, small synthetic DNA molecules termed oligonucleotides, are introduced in plant cells. The plant's natural repair mechanism may use these molecules as a repair template, copying any modification in the oligonucleotide compared to the native sequence into the plant genome. This results in plants in which the target DNA sequence is altered as desired. The oligonucleotides themselves are not incorporated into the genome.

The technique delivers the DNA fragments into protoplasts. Regeneration of plants from protoplasts is a demanding technique and is only successfully applied to a limited number of crops. ODM is applied for gene editing aimed at knocking-out or adapting gene function in crops.

As for SSN-1 and SSN-2, mutations induced by ODM may also be obtained using traditional mutagenesis methods. The benefit of ODM technology over traditional mutagenesis is that it does not produce thousands of other mutations. Apart from the time it takes to remove these unwanted mutations, it is also impossible to trace and remove them all, a problem that is overcome with ODM. Herbicide tolerance is the most advanced trait obtained by ODM. Crops developed with ODM are not on the market yet. However, herbicide tolerant canola with resistance to imidazolinone herbicides generated using ODM has received authorization by the 'Canadian Food Inspection Agency' and 'Health Canada' to be commercialized².

2 www.producer.com/2014/03/growers-to-see-new-ht-canola-in-2016

Oligonucleotide-directed mutagenesis (ODM)

Oligonucleotide-directed mutagenesis (ODM) is partly similar to SSN-2 as also here small repair templates are introduced in plant cells, and plants are selected in which a modification in the template is copied into the DNA. The difference is that no gene construct is introduced into the plant to induce a DNA break. The technique relies on spontaneously occurring DNA correction activity. Mutations resulting from ODM can also be obtained using traditional mutagenesis techniques. The benefit of ODM technology over traditional mutagenesis is that it does not produce thousands of other mutations. Apart from the time it takes to remove these unwanted mutations, it is also impossible to trace and remove them all, a problem that is overcome with ODM.

RNA-dependent DNA methylation

Plants have an innate defence mechanism using the 'RNA-induced silencing complex' (RISC) to recognise foreign DNA, for instance originating from viruses, and block its transcription. RISC is triggered by short double-stranded RNA molecules (which may be degradation products of viral origin), and then attaches methyl groups to the matching DNA sequence, which inactivates (expression of) the DNA (Fig. 4).

RNA-dependent DNA methylation (RdDM)

Plants have a defence system to protect themselves against foreign DNA, for instance from viruses. This system is called the RNA-induced silencing complex (RISC). This system is activated by short double stranded RNA molecules, since these may be degradation products of viral origin. The RISC system forms a complex with the foreign RNA and locates the DNA matching the RNA. Next, methyl groups are attached to the foreign DNA and these methyl groups block the expression of genes.

For application of RdDM as new breeding technique, recombinant genes that produce RNA molecules that match the target gene or its promoter region are designed and introduced into the plant cell. After production, the RNA molecules will be recognized by the RISC system resulting in methylation of the corresponding DNA. As a result the expression of the gene is blocked.

Methylation does not change the DNA sequence itself.

RNA-dependent DNA methylation (RdDM) uses the plant's RISC system for silencing the expression of specific plant genes. For RdDM, recombinant genes are designed which contain fragments of the natural gene to be targeted. When

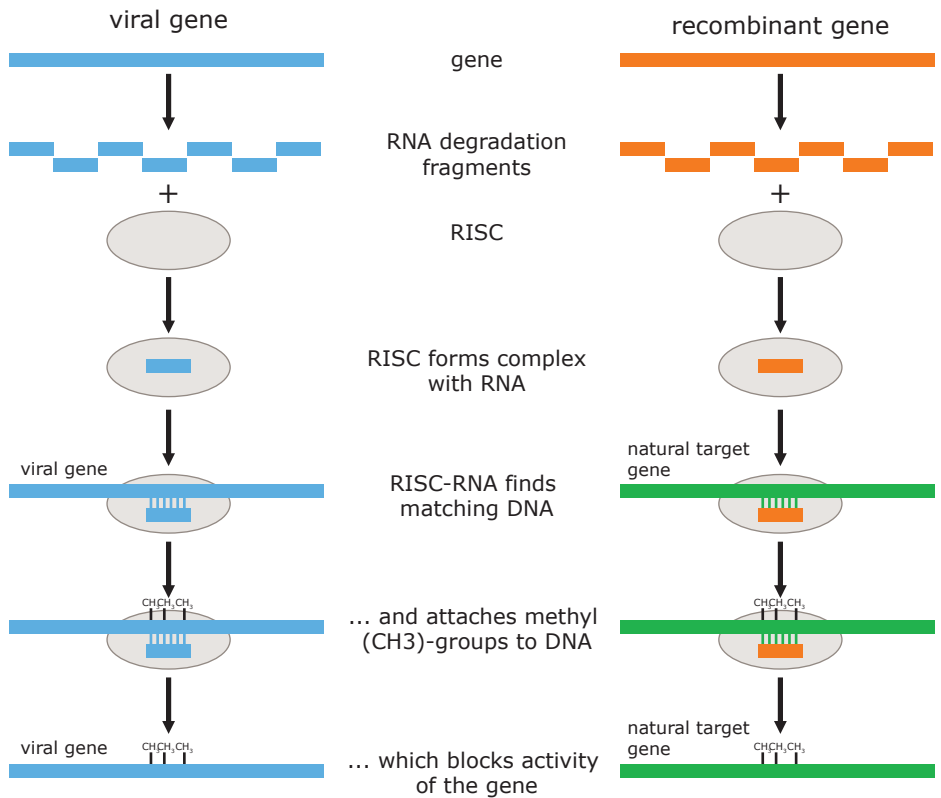


Figure 4 Simplified graphical representation of RdDM. On the left side the natural defence system leading to methylation of a viral gene is shown. On the right side recombinant-derived RNA molecules guide the RISC to its natural counterpart resulting in DNA methylation and subsequent block of gene activity. The recombinant gene is containing fragments of the natural gene to be targeted.

the recombinant genes are introduced into the plant cell, short RNAs will be produced which induce RISC-mediated methylation of the plant gene or its promoter, which then blocks expression of the plant gene. Because RdDM changes the methylation status of the DNA rather than the DNA sequence itself, it is called an epigenetic modification. After removal of the recombinant genes, the local methylation of the DNA remains in place, but the DNA sequence of these plants is identical to the parental plants. The methylation status is not stable and tends to fade out over generations. There are no examples of application of RdDM known aimed at potential commercial application.

Reverse breeding

A heterozygous offspring plant that is selected from a breeding program cannot be reproduced from seeds, as the unique combination of genetic variation will be lost upon further seed multiplication or reproduction. It therefore needs to be reproduced vegetatively. Seed companies aim however at reproducing elite cultivars from seed.

A strategy, called reverse breeding, was designed by the company Rijk Zwaan to create parental lines to produce the selected heterozygous offspring plant from seed. These parental lines are both homozygous, having no variation within their own set of chromosomes (see Fig. 5). The genetic variation between both parental lines is however large and together they contain all the genetic variation that is desired in the hybrid offspring plant. Because the parents are homozygous, all offspring plants will have the same genetic constitution. Also the parental lines can be stably multiplied through seed.

Reverse breeding makes use of a GM step to introduce recombinant genes that suppress meiotic recombination (exchange of genetic information between chromosomes pairs) during production of gamete cells (egg cells and pollen). These gamete cells, which contain a single, non-recombined copy of each chromosome, are then subjected to a specialized tissue culture technique called doubled-haploid production, to regenerate homozygous plants. Crossing two of these doubled-haploid plants that have a complementary set of chromosome pairs (the homozygous parental lines), will produce uniform seeds of hybrid offspring identical to the heterozygous plant of commercial interest.

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A GM step to suppress recombination of chromosomes and specific tissue culture steps are used to create these parental plants.

In the final reverse-breeding steps the genes used for the genetic modification are crossed out, resulting in end-products that are free of GM-related DNA sequences.

Because during doubled-haploid production plants are selected that did not inherit the recombinant genes, the hybrids produced through reverse breeding are identical to the plant it started with.

Reverse breeding has been demonstrated in the model plant *Arabidopsis thaliana* (thale cress; Dutch: zandraket) but the technique is still far from application in commercial crops.

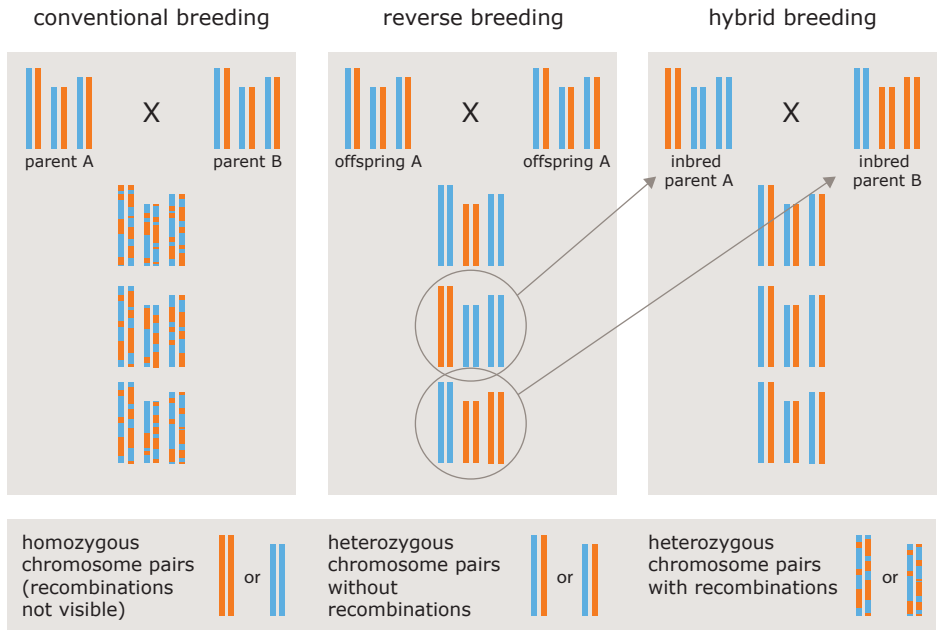


Figure 5 Schematic overview of final outcome of different breeding approaches. During conventional breeding recombination of chromosome pairs results in reshuffling of genetic material and unique combination of genetic variation will be lost. In reverse breeding a selected heterozygous offspring plant is crossed with itself, while chromosomal recombination is suppressed by a transgene and results therefore in lines with homozygous chromosome pairs. The haploidization step, this is producing plants in which only one chromosome of each chromosome pairs is present, and the subsequent doubling of these chromosomes again, so that doubled-haploid plants with homozygous chromosome pairs are produced, are not shown here (explanation of homozygous vs heterozygous chromosome pairs is shown at the bottom box). For hybrid variety production, parental lines in which the genetic variation of the chromosome pairs complements each other are selected from the reverse breeding program. Crossing such lines will result in uniform offspring hybrid plants (seeds), which are genetically similar to the plants with which the reverse breeding was started with.

Induced early flowering

(Fruit) trees generally have a long juvenile phase. For example, in apple it takes five or six years before the first flowering occurs. This is a serious constraint for creating new apple cultivars through crossing and selection. In the case of introduction of e.g. a new disease resistance gene from a wild apple species, four to five generations are necessary to obtain apples of good size and taste. In the case of the apple scab disease resistance gene (apple scab is an important disease in apple culture caused by the fungus *Venturia inaequalis*), this process took 50 years.

In the induced early flowering approach recombinant genes that promote flowering in the first year are introduced into a plant. The early flowering genes used originate from *Arabidopsis* (thale cress, a lab model plant) and are introduced into the plant by genetic modification using *Agrobacterium*. Using the transgenic early flowering trees (see Fig. 6), one breeding cycle can be performed in a year. In the final breeding step the recombinant early flowering genes are crossed out resulting in varieties that are free of GM (transgenic) sequences. These varieties are indistinguishable from varieties obtained through conventional breeding, but can be obtained decades earlier. The successful application of this new breeding technique has been demonstrated in an early flowering-based apple breeding programme in Europe in which pyramiding of various resistance genes from two different parents was achieved in two years.

The method is commercially used in plum breeding in the US (known as 'FasTrack'). Regulators at the USDA decided that plum cultivars resulting from the FasTrack breeding system will be outside of the agency's regulatory authority, as long as those cultivars do not contain transgenes or pieces of transgenes.

Induced early flowering

In the induced early flowering approach a recombinant gene that induces flowering is introduced into a woody plant. The gene results in flowering of the seedlings in the first year, and thus speeds up breeding in species with a long generation time (e.g., 5 or 6 years in apple). In the final breeding step the recombinant early flowering genes are crossed out resulting in varieties that are free of GM sequences.



Figure 6 Early flowering transgenic apple seedling in which an early flowering gene from *Arabidopsis* is introduced (from Flachowsky et al.)

Grafting on a GM rootstock

Grafting is a method where the top part of one plant (the scion) grows on a rooted lower part of another plant (the rootstock). Grafting was invented by the Greek in the 4th century BC and it is still instrumental in the large-scale cultivation of vegetatively propagated fruits (e.g. apples, grapes, roses). It is now also used in vegetables (tomatoes, cucumbers) as a means to improve the performance of the root system.

Using genetic modification the characteristics of a rootstock, such as rooting ability on heavy soils or resistance to soil-borne diseases, can be improved. Such an improvement of the rootstock will eventually lead to a better performance of the scion. When a non-GM scion is grafted on such a GM rootstock, the resulting combined plant will be regarded as GM, but products (such as fruits or flowers) harvested from the scion do not carry the genetic modification. There are currently no examples known of use of grafting on a GM rootstock that have a potential commercial application.

Grafting on GM rootstock

The top of a non-GM plant is grafted on a GM rootstock. Harvested products from the shoot are GM free. This technology is used in fruit, vegetable and tree crops.



Examples of potential application of New Plant Breeding Techniques

Example 1

Late blight (*Phytophthora*)-resistant potato using cisgenesis

Potato is the fourth crop worldwide and it is an important source of food and starch. The most devastating disease in potato is 'late blight' caused by *Phytophthora infestans*, which in agricultural practice is controlled by a frequent application of fungicides. In the 'gene pool' that is available for potato breeding various *Phytophthora*-resistance genes are present. However, breeding in potato is difficult because of its complex genetic structure. For example, after introduction of a single resistance gene from a wild species it took almost 50 years to breed a *Phytophthora*-resistant potato variety with good quality characteristics. Resistance of cultivars with resistance based on a single gene may easily be broken by the rapidly evolving *Phytophthora infestans*. Cisgenesis is a promising alternative to achieve a more rapid improvement in potato. At Wageningen UR cisgenesis has been applied for the introduction of a combination of three different *Phytophthora* resistance genes originating from three different wild potato relatives into a commercial potato cultivar. The performance of the cisgenic potato lines is currently under evaluation in field trials. Stacking of resistance genes through cisgenesis is the most advanced technique at the moment for achieving *Phytophthora*-resistance in potato. Commercialization of cisgenic *Phytophthora*-resistant potato lines would be implementable in a time-frame of 5-10 years.

Example 1: Phytophthora resistant potato using cisgenesis

Cisgenesis has been applied in potato to combine three different *Phytophthora* resistance genes into a commercial potato cultivar within a few years. Introduction of a single resistance gene took almost 50 years through traditional breeding. Resistance based on a single gene was easily broken.

Haverkort, A.J. et al. (2009) Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. *Potato Res.* 52, 249-264

Example 2

Bacterial leaf blight resistance in rice using genome editing (by TALENs, a specific form of SSNs)

Rice is the major staple food for a large part of the world's human population. In the majority of the rice-growing countries bacterial leaf blight in rice caused by *Xanthomonas oryzae* pathovar *oryzae* (commonly known as Xoo) is the most widespread pest. Several resistance genes have been identified and utilized for breeding of resistant varieties, but resistance of cultivars may easily be broken by the rapidly evolving pathotypes of Xoo. Therefore the discovery of new wider-spectrum resistance genes and pyramiding (stacking) of several resistance genes are important issues, as in the potato example above. In 2012 TALENs have been employed to create a novel resistance towards bacterial leaf blight in rice based on the rice bacterial blight-susceptibility (S) gene, Os11N3. This rice gene is hijacked by the Xoo pathogen and has become essential for the pathogenesis of several Xoo lines. Xoo secretes effector molecules that bind to the promoter of the Os11N3 gene and induce strong expression of this gene for the benefit of Xoo's infection process. Deletion of the promoter binding site for the Xoo effector molecules by specifically designed TALENs (SSN-1) prevented activation of the Os11N3 gene by Xoo and resulted in strong resistance to infection by Xoo pathotypes in rice, while other functions of the Os11N3 gene for the plant remained unaffected. Subsequent removal of the TALENs recombinant DNA using genetic segregation through crossing produced bacterial leaf blight resistant rice offspring plants that were only different from the original cultivar by a deletion of 5-10 basepairs at the targeted location in the Os11N3 gene promoter. This minimal deletion did not change the gene expression pattern during rice development.

Natural variation in the Os11N3 gene that would prevent induction by Xoo strains has not been identified in the rice germplasm.

Example 2: Bacterial leaf blight resistance in rice using SSN-1

Rice has a gene that encodes its susceptibility to a bacterial disease causing leaf blight. With TALENs, a specific SSN technology, a small piece from the promoter regulating expression of that rice gene was removed. This resulted in resistance to infection by specific bacterial pathotypes in rice causing leaf blight. The resistance could not have been obtained with traditional breeding.

Li, T. et al. (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* 30, 390-392

Example 3

Powdery mildew resistance in wheat by genome editing (using TALENs and CRISPR-Cas9)

A recent (July 2014) publication describes SSN-1-mediated mutation of the susceptibility gene called *mlo* in bread wheat leading to resistance to powdery mildew, using TALENs as well as CRISPR-Cas9. This S-gene from wheat is required for the infection process of powdery mildew. Because bread wheat has three genomes, it has different copies of each gene and therefore, it is difficult to achieve complete knock-out of all genes by random mutagenesis. Multiple TALENs and/or CRISPR-Cas9 proteins were used to mutate the three copies of the *mlo* gene in the same plant simultaneously.

Example 3: Powdery mildew resistance in wheat using SSN-1

SSN-mediated mutation was used to knock-out the expression of the susceptibility gene *mlo* in bread wheat. Silencing of this gene led to resistance to powdery mildew.

Wang, Y.P. et al. (2014) Simultaneous editing of three homoeoalleles in *hexaploid* bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947-951

Example 4

Improving oil quality by genome editing (by TALENs)

A recent (May 2014) publication describes metabolic engineering of genes that code for enzymes in a biochemical pathway of oil in soybean using TALENs. In soybean an enzyme called fatty acid desaturase converts the mono-unsaturated (omega-9) fatty acid oleic acid into the polyunsaturated fatty acid linoleic acid. Unsaturated fatty acids are considered healthy, but high levels of polyunsaturated fats are undesirable in oils for consumption. Mutation of the gene coding for fatty acid desaturase using TALENs (SSN-1) resulted in soybean lines that produce oil low in polyunsaturated fat. This oil has a better quality for human consumption. Such a mutation may also be found naturally, but it is not known from soybean germplasm yet.

Example 4: Improved oil quality using SSN-1 (TALENs)

SSN-mediated mutation was used to knock-out the function of a gene in the biosynthetic pathway for fatty acids in soybean. This resulted in soybean lines that produce oil that is higher in mono-unsaturated fatty acids and lower in polyunsaturated fatty acids. This oil has an improved quality for human consumption.

Haun, W. et al. (2014) Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol. J.* 12, 934-940

Example 5

Resistance to AHAS (ALS)-targeting herbicides (in various crops) using ODM

Herbicides that inhibit the plant acetohydroxyacid synthase (AHAS) enzyme (also known as acetolactate synthase (ALS)) are among the most widely used in the world. The AHAS enzyme is necessary for the production of essential amino acids in the plant and inhibition of AHAS by herbicides leads to plant metabolic inhibition and death. Single amino acid substitutions at either of two crucial positions in the AHAS enzyme result in a herbicide-insensitive form of this enzyme and consequently in a herbicide-tolerant plant phenotype. The use of ODM to create herbicide-tolerant plants has been applied to different crops. For example maize plants tolerant to imidazolinone herbicides have been engineered through targeted modification of endogenous AHAS genes using a combined RNA/DNA oligonucleotide. A precise single-point mutation was copied into the maize AHAS gene, at a position known to confer imidazolinone tolerance. Both BASF and Cibus LLC applied ODM for the targeted mutation of AHAS to create herbicide-tolerant canola and rapeseed varieties. BASF sees ODM as a preferred method to introduce herbicide tolerance in canola and rapeseed because of the shorter development time when compared to mutagenesis. In the UK, the Advisory Committee on Releases to the Environment (ACRE), who advises the UK Government, considered that herbicide-tolerant oilseed rape plants produced by Cibus LLC have been developed using a technique which does not involve the use of recombinant nucleic acid molecules. In Germany, the competent authority recently considered the similar herbicide-tolerant oilseed rape to be outside the scope of German GM regulation ("Gentechnikgesetz"), under the provision that the decision will be withdrawn when the European Commission would decide otherwise under Directive 2001/18/EC³.

Example 5: ALS herbicide-tolerant crops using ODM

A point mutation was introduced into the maize AHAS gene, which changed a single amino acid in the protein at the position known to confer imidazolinone tolerance, using combined RNA/DNA oligonucleotides. ODM is seen as a preferred method to introduce herbicide tolerance in canola and rapeseed because of the shorter development time compared to mutagenesis.

Zhu, T. et al. (2000) Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nat. Biotechnol.* 18, 555-558

3 www.transgen.de/pdf/zulassung/BVL_ODMCibus.pdf

Example 6

Induction of early flowering in trees

For the introduction of new traits, such as disease resistances that are present in wild apple species, into modern cultivars three or more generations are required to remove undesired characteristics that are inherited from the wild apple.

It may take 50 years to obtain apple cultivars expressing the new traits and at the same time having a fruit quality that can compete with the world's leading cultivars.

With genetic modification, successful early flowering has been obtained through overexpression in apple of the *Arabidopsis* 'Flowering Locus T' (*AtFT*) gene, which induces flowering in apple seedlings within the first year. These early flowering transgenic apple lines were then used for a so-called "fast breeding" programme for gene introduction. In year 1 a selected early flowering transgenic apple line was crossed with the fire blight-resistant wild species *Malus fusca*. Transgenic early flowering F1 seedlings were then selected and backcrossed in year 2 with other apple lines that have apple scab and powdery mildew resistance genes and additional fire blight resistance genes. Seedlings of this cross were screened in year 3 with genetic markers to identify those plants that have inherited all three disease resistance genes, but not the early flowering gene.

Example 6: Early flowering in trees using a temporary transgenic approach

Using genetic modification the *Arabidopsis* 'Flowering Locus T' (*AtFT*) gene was introduced in apple. Overexpression of *AtFT* resulted in induction of flowering in apple seedlings within the first year.

The early flowering transgenic apple lines were used in a so-called "fast breeding" program. In the first year fire blight resistance genes were introduced by crossing. The resulting early flowering seedlings were crossed in the second year with a line with apple scab and powdery mildew resistance genes. From this cross seedlings were selected in the third year which had inherited all three disease resistance genes, but not the early flowering gene. The selected seedlings were therefore free of GM-related sequences.

Flachowsky, H. et al. (2011) Application of a high-speed breeding technology to apple (*Malus x domestica*) based on transgenic early flowering plants and marker-assisted selection. *New Phytol.* 192, 364-377

A similar fast-breeding scheme is reported for plum and was called "FasTracking" Plum Breeding. By transforming a Flowering Locus gene into European plum (*Prunus domestica* L.), transgenic plants were produced that flowered and produced fruits in the greenhouse within 1 to 10 months after germination.

Regulators at the USDA decided that plum cultivars resulting from the FasTrack breeding system will be outside of the agency's regulatory authority, as long as those cultivars do not contain transgenes or pieces of transgenes.

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The following scientists were involved:

Jan Schaart, Marleen Riemens, Clemens van de Wiel, Bert Lotz, René Smulders.

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