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Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants

Evert Jacobsen[1,3] and Henk J. Schouten[2]

[1] Wageningen University and Research Centre, Laboratory of Plant Breeding, P.O. Box 386, 6700 AJ Wageningen, The Netherlands
[2] Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[3] Transforum Agribusiness & Rural Areas, Louis Pasteurlaan 6, 2700 AB Zoetermeer, The Netherlands

There are two ways for genetic improvement in classical plant breeding: crossing and mutation. Plant varieties can also be improved through genetic modification; however, the present GMO regulations are based on risk assessments with the transgenes coming from non-crossable species. Nowadays, DNA sequence information of crop plants facilitates the isolation of cisgenes, which are genes from crop plants themselves or from crossable species. The increasing number of these isolated genes, and the development of transformation protocols that do not leave marker genes behind, provide an opportunity to improve plant breeding while remaining within the gene pool of the classical breeder. Compared with induced translocation and introgression breeding, cisgenesis is an improvement for gene transfer from crossable plants: it is a one-step gene transfer without linkage drag of other genes, whereas induced translocation and introgression breeding are multiple step gene transfer methods with linkage drag. The similarity of the genes used in cisgenesis compared with classical breeding is a compelling argument to treat cisgenic plants as classically bred plants. In the case of the classical breeding method induced translocation breeding, the insertion site of the genes is *a priori* unknown, as it is in cisgenesis. This provides another argument to treat cisgenic plants as classically bred plants, by exempting cisgenesis of plants from the GMO legislations.

Developments in classical and modern plant breeding

At the onset of plant breeding, sufficient genetic variation was available, and the breeder did not need many crosses, seedlings or selection schemes to obtain new varieties that were more domesticated than the existing ones. However, the occurrence of (a)biotic stress and the need for quality traits, requires more genetic variation and stimulated mutation breeding for the direct improvement of existing varieties, or for crosses with wild species and consecutive backcrosses, for improved breeding material containing novel traits. This, more complex, breeding approach with wild species, called introgression breeding, has been worked out in detail for many crops [1], and there are many successful examples of the domestication of genes coding for new traits [2]. In some important interspecific crosses, suppression of recombination occurred, and this required induced translocations by induction of mutations; this was followed by backcrosses [2,3].

Biotechnology has broadened these approaches by adding *in vitro* techniques, such as embryo rescue, regeneration and protoplast fusion; genomics – for the development of genetic maps with molecular markers, enabling marker assisted selection (MAS) and comparative genomics [1,4]; and genetic modification (GM).

Therefore, existing varieties can also be improved by genetic modification using transgenes and, more recently, cisgenes originating from the crop plant itself or from crossable species [5,6]. Worldwide, strict rules accompany the GM approach; these are primarily based on transgenes representing the new gene pool. However, isolated cisgenes from the gene pool of the classical breeder are increasingly becoming available, in combination with marker-free transformation protocols. Therefore, we argue that the risks of cisgenes are comparable with those in classical breeding, and comparisons with classical breeding approaches have been made to classify cisgenesis and transgenesis (Box 1).

Mutation breeding

Mutations are the ultimate source of genetic variation, and together with selection and genetic recombination, the most important factors in evolution. Mutations at the gene level are mostly recessive and, by definition, single-cell events; therefore, they are always accompanied with chimerism. This problem, and that of making recessive mutations homozygous, can be solved in seed propagated crops through the sexual cycle, but this is not possible in heterozygous, vegetatively propagated crops [7]. Therefore, in the autotetraploid potato, for example, only a few mutations (tuber color, tuber shape and amylose-free starch) have been used for breeding purposes [7]. However, in diploid apple the occurrence of spontaneous mutations leading to improved cultivars are frequently found, as shown in the skin color- and growth rate-altered mutants of the cultivars Golden Delicious, Jonagold and Elstar [8]. Many seed propagated crops with homozygous parents, such as barley, rice, tomato,

Corresponding author: Jacobsen, E. (evert.jacobsen@wur.nl).
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Box 1. Comparison of introgression breeding, induced translocation breeding and cisgenesis.

(a) Introgression breeding is interspecific hybridization between a receptor plant and a donor plant, followed by backcrosses with the recipient plant and simultaneous selection for the trait of interest. The outcome is introgression of the target gene into the recipient, with linkage drag from the donor. The amount of linkage drag is dependent on the (reduced) frequency of crossover events around the target gene, number of backcrosses, and the ease of selection against genes with negative side effects. MAS can reduce linkage drag problems, as shown in new aphid-resistant lettuce varieties (*Lactuca sativa*), by removing the tightly linked CRA (compact growth and rapid aging) phenotype in the donor DNA (from *Lactuca virosa*) [21]. Introgression breeding is frequently successful in self-fertilizing diploid crops, but linkage drag problems are frequently found in more complex and in vegetatively propagated crops.

(b) Induced translocation breeding starts as introgression breeding; the big difference is the absence of crossing-over because of lack of pairing of homologous chromosomes of the donor and recipient plants. After backcrosses and selection for resistance, addition lines are obtained containing the donor chromosome with the target gene. Following γ-radiation of the seeds and selection in the following generations, plants can be found with the target gene containing the translocation but which lack the majority of the remaining donor chromosome linked to the target gene. The frequency of such plants is low, and the insertion place of the translocation in the receptor is random – that is, on different chromosomes. The inserted R-genes posses varying numbers of neighboring genes from the donor; therefore, a restricted number of translocation events are the basis for further breeding. The removal of negative linkage drag cannot be easily realized by additional backcrossing. Negative side effects from individual insertions can only be overcome by selecting for new insertions or by compensation breeding. Examples of such translocations are shown in Table 1. For wheat, an overview of 57 translocations has been made, including cytological observations by C-banding and *in situ* hybridization [3].

(c) In the cisgene approach, sufficient numbers of marker-free transformants with random and single T-DNA insertions, and sufficient expression of the cloned cisgene in the recipient, are produced. The next step is the selection of plants in the growth chamber, glasshouse and field, respectively. Linkage drag with other genes from the donor species is absent, and selection of plants with the best performing gene insertions and minimal negative side effects is made in the field (Figure I).

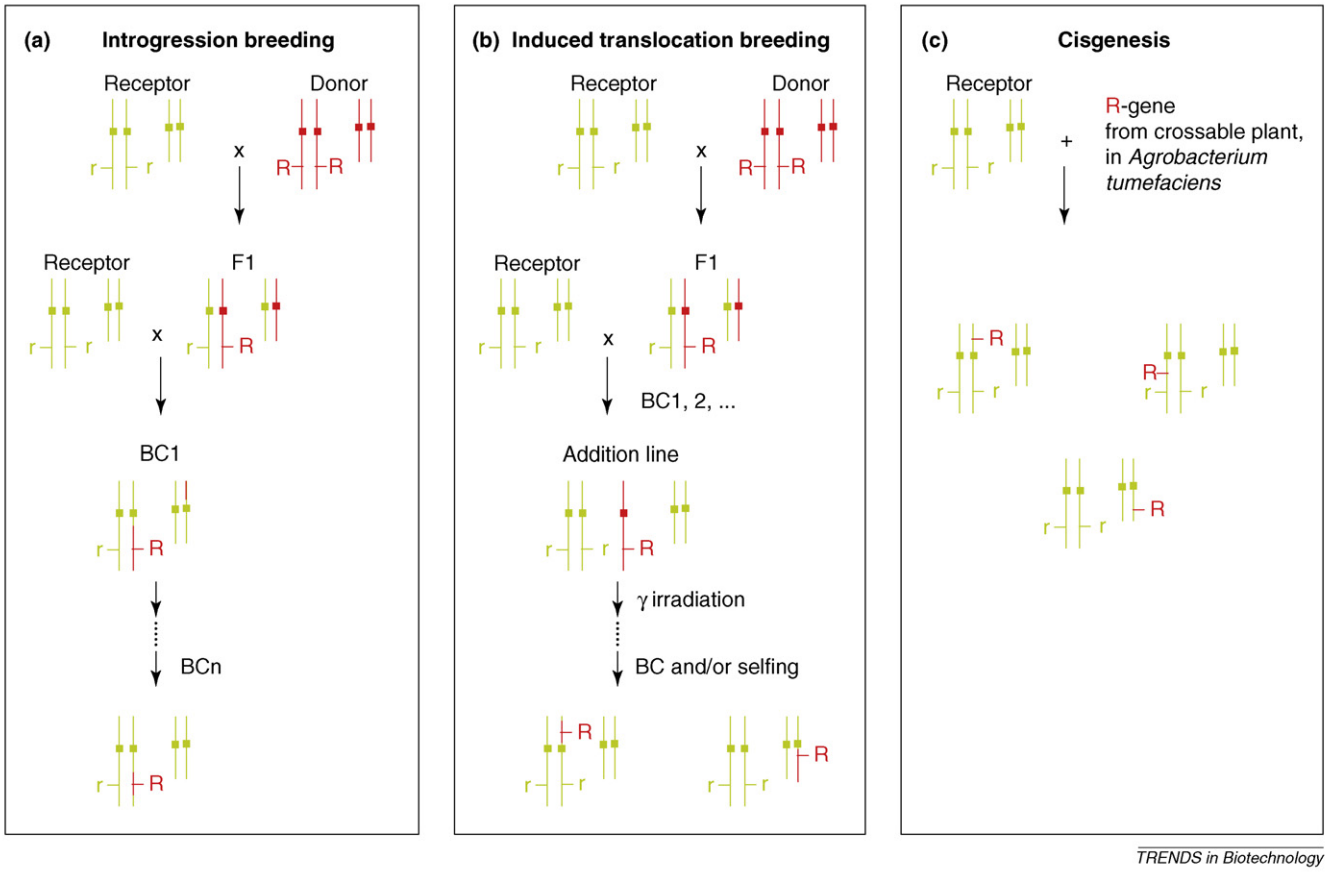

Figure I. The most important steps in introgression, translocation and cisgenic breeding. These result in **(a)** insertion, **(b)** translocation of the R-gene insertion with linkage drag (two examples), and **(c)** cisgenic R-gene insertion without linkage drag (three examples).

maize, Brassica sp. and petunia, arose from induced mutations: mutation breeding has resulted in >2250 varieties [9]. Various types of mutations are possible: intragenic; intergenic; and genome changes (polyploidy, haploidy and aneuploidy). At the DNA sequence level, three types of mutations can be discerned: base substitutions; frameshifts; and macro-mutations, such as inversions, deletions, duplications, (induced) translocations and insertions by natural transposons [7]. These insertions are highly comparable with T-DNA insertions in GM-plants. Two of the main differences between genetic modification and mutation breeding are the dominant inheritance of the introduced trait, in the case of genetic modification, compared with the usually recessive inheritance of mutations,

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and the higher level of damage to chromosomal DNA during mutation induction, followed by repair [7].

Introgression breeding

The need for biodiversity beyond that of a wild species has increased, to feed plant breeding with new sources of natural genetic variation; however, the use of wild species is a considerable resource for extending genetic variation in plant breeding. The detection of centers of origin of crop plants, the expeditions to collect germplasm, the foundation of gene banks, the evaluation of collected plant material, and the research related to interspecific hybridization – such as resynthesis of allopolyploids, synthesis of new allopolyploids (Triticale) and autotetraploids (ryegrass) and introgression breeding – have contributed to the enlargement of genetic variation [1,10]. The use of genetic variation found in gene banks by intra- and interspecific crosses, and consecutive backcrosses with appropriate selection methods, are the heart of modern plant breeding [1,11].

One of the drawbacks of introgression breeding in more complex heterozygous crops, such as potato and apple, is the long-lasting process of interspecific hybridization and consecutive backcrosses with the cultivated species under the simultaneous selection for the trait of interest [12]. The usefulness of the gene of interest is highly dependent on linkage drag, which is the genetic linkage of the gene of interest to genes that can have a negative impact. This phenomenon can frequently be overcome by meiotic crossing-over; however, crossing-over cold spots can be found around introduced genes that prevent the removal of genes with a negative impact from the donor species [11]. There are several examples in the literature showing that desired traits from related species were not available for variety breeding because of linkage with genes coding for late maturity, small fruits or other negative traits, or compensation breeding was needed [13,14]. Where introgression breeding is successful, the final result is always introgression of the target gene surrounded by many donor genes (Box 1).

Induced translocations

Random insertion of alien genes by induced translocations is normal practice in plant breeding (Box 1). In allopolyploid wheat, introgression breeding with diploid wild species is complicated because of the *Ph* gene on chromosome 5B, which prevents meiotic crossing-over between homologous chromosomes [13]. Introgression breeding with the wild species led, in these cases, to addition or substitution lines, with the complete donor chromosome containing the gene of interest. One way to overcome this introgression problem is γ-radiation of the seeds of addition or substitution lines, leading to chromosome breakages and random insertion of a piece of donor chromosome with the desired gene in one of the receptor chromosomes [3,13]. The place of insertion is random, and the translocation was accompanied with linkage drag. The same radiation approach on seeds has also been applied in tobacco, sugar beet, oat and radish (Table 1). In oat, addition lines with individual maize chromosomes [14] were made by induced translocations, for dissecting the maize genome. In polyploid blackberry, natural translocations have been used [15]. Natural translocations have also been visualized in molecular genome evolution studies of different genera [4,16].

Table 1. Examples of gene transfer by random insertion using induced translocation breeding by irradiation

Crop	Source	Trait	Ref
Wheat	*Aegilops umbellulata*	Brown rust	[3]
Wheat	*Secale cereale*	Mildew and brown rust	[3]
Wheat	*Agropyron elongatum*	Brown and black rust	[3]
Wheat	*Agropyron intermedium*	Brown and yellow rust	[3]
Wheat	*Aegilops speltoides*	Brown rust	[3]
Oats	*Avena barbata*	Mildew	[3,25]
Beet	*Beta patellaris* *Beta procumbens*	Nematodes	[26]
Tobacco	*Nicotiana glutinosa*	Tobacco mosaic virus	[27]
Radish	*Brassica rapa*	Spread leaf type	[28]

Transgenes and GMO regulations

Transgenes originating from non-crossable species, such as bacteria and viruses, were the main gene source at the onset of GM breeding of plants [17]. Furthermore, bacterial genes coding for resistance to antibiotics or herbicides were needed for the selective growth of the transformed cells. Genetic modification was the domestication of transgenes into the crop plant and its agro-ecosystem. Therefore, European regulations [17], as well as many other regulations for the introduction of GM crops into the environment and onto the market, have been conditioned by transgenes. These transgenes were a novelty in plant breeding, providing a new gene pool, and the regulations developed for GM crops at all stages of variety development are only focused on the prevention and control of risk using transgenes. Therefore, the release of GM crops is loaded with risk assessments for genes from the new gene pool. A new situation has been created by the availability of isolated cisgenes, which have a known history of safe use in classical plant breeding.

Cisgenesis

Cisgenesis is the introduction of isolated genes with their native promoters from crossable species or from the crop plant itself [5,6]. The main tools for cisgenesis in plants are the new transformation protocols, without (bacterial) selection markers, in seed propagated crops by co-transformation [18] and in vegetative propagated crops by marker-gene-free vectors or removable marker genes [19,20]; and the isolated genes from crop plants and their crossable relatives. Cisgenesis was born from the combination of these. In practice, cisgenesis can be applied for resistance and quality breeding of all crops, but particularly in vegetatively propagated heterozygous crops, such as potato and apple. In many cases, resistance genes are found in related wild species, and introgressions or induced translocations are needed for their introduction into cultivars. This approach generates an accumulation of linkage drag problems if the stacking of genes is needed. Another observation in resistance breeding is that resistance genes are found in clusters. When several resistance genes from the same cluster on different homologous chromosomes must be combined, a crossing-over event within the cluster

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is needed. This event is rare; however, in some cases, it is feasible with the aid of MAS, as shown for insect-resistant lettuce [21]. The linkage-drag-free cisgenic approach is highly attractive, and embodies the possibility to stack resistance genes from different sources, even if they originate from the same chromosomal position in different species or accessions, as described for *Phytophthora*-resistant potato [12].

Insertion of cisgenes is random and comparable with random insertion in transgenic GM varieties, natural transposons and induced translocations [4,13]. These random insertions in different transformants are important (Box 1) because they enable the selection of insertion places with the best expression of the cisgene with minimal side effects – most of the cisgenic plants with major side effects are removed in the growth chamber and glasshouse. The intensive selection process in the field, at multiple sites, will eradicate genotypes with minor but agriculturally important side effects, including possible biochemical changes such as higher toxic content. Determination of the glycoalkaloid content is routine in the classical breeding of potato [11]. When comparing cisgenesis with induced translocation breeding, cisgenesis can be regarded as induced microtranslocation breeding.

Cisgenes and GMO-regulations

Polyploidization and *in vitro* fertilization are not considered GM techniques [17]; however, in European legislation, mutation breeding and protoplast fusion are [17]. In spite of this, mutation breeding is exempted from this regulation, and fusion of plant cells is exempted if the cells are from crossable plants [17]. Neither are mutation breeding and cell fusion of crossable plants part of the Cartagena protocol on biosafety [22] (http://www.biodiv.org/biosafety/default.aspx). Conversion of individual chromosomes or chromosome arms, such as additions, substitutions, or parts thereof, as observed in, for example, wheat breeding with the 1BL/1RS translocation of rye [23], is not even regarded as GM in the regulations. Therefore, it makes sense that cisgenesis of individual genes coding for agricultural traits, representing only one or a few alleles of the same or crossable species, should be exempted from the GMO regulation. Cisgenesis provides no extra risks compared with, for example, induced translocation breeding or mutation breeding. Moreover, cisgenesis avoids linkage drag, and therefore, prevents risks from unknown hitchhiking genes [24].

Conclusion

Despite their successes, the classical methods of alien gene transfer have disadvantages and difficulties, particularly linkage drag, that require time-consuming backcrosses and simultaneous selection steps. Cisgene microtranslocation is a powerful alternative: it is a single-step gene transfer without linkage drag; insertion-related side effects can be overcome by normal selection; stacking of (resistance) genes as a strategy is more feasible; and existing varieties can be improved directly using genes from the gene pool of breeders.

In our laboratory, we produce cisgenic apple varieties by stacking resistance genes from crossable *Malus* plants for durable resistance to apple scab, with the aim of environmentally friendly fruit production. Furthermore, long-term resistance research in the potato, *Phytophthora infestans*, and its interaction with R-genes is ongoing, to produce proof-of-principle for the creation of a cisgenic potato with sustainable resistance [12].

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