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CISGENESIS – An alternate approach for development of genetically modified crops

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ABSTRACT

Intragenic modification in the context of other plant breeding of desired traits from wild germplasm is seriously affected by linkage drag. It requires several generations of breeding and very time consuming, especially for long generation period crops and wild sources. These problems would be prevented if only the gene of interest would be added, leaving the undesired genes in the wild germplasm behind. This is feasible by means of 'cisgenesis'. Cisgenesis/Intragenesis deploy the same technology as transgenesis. A cisgene contains its native introns and is flanked by its native promoter and terminator in sense orientation. Cisgenic plants contain solely genes that have been present in the conventional breeder's germplasm. Consequently, cisgenic plants have no extra risks compared to plants from conventional breeding or mutation breeding. Therefore we propose that cisgenic plants are exempted from the burden of the GMO regulation. An additional advantage of cisgenesis is that the genetic makeup of the established cultivars with a history of safe use is maintained. Only a few genes are added. In case of self-incompatible, heterozygous crops it is impossible to add genes and restore the genetic makeup through cross breeding. The knowledge of functions and DNA sequences of plant genes of plants is increasing very rapidly. Cisgenesis is a valuable approach for valorizing this knowledge. We apply cisgenesis to apple and potato in order to obtain polygenic durable resistance to apple scab and Phytophthora infestans respectively. Also we have introduced the MdMYB10 transcription factor from apple that upregulates the anthocyanin pathway, leading to red-fleshed apples

INTRODUCTION

The release of genetically modified (GM) crops into agricultural production has raised considerable debate, especially among the general public, politicians and bureaucrats (1, 2). One of the main underlying concerns is the transfer of genes across very wide taxonomic boundaries (3). Public opinion surveys on genetic modification issues have repeatedly found that gene transfer within species is a more acceptable approach. This response has been consistent across societies throughout the world, including New Zealand (4), North America (5), and Europe (6). One approach to address many of the ethical, religious and/or public concerns in the genetic engineering debate is the transfer of genes within the gene pools currently available to plant breeders, referred to as intragenic (7), all-native (8) or cisgenic (9). However, such gene transfer still relies on vector systems based on DNA from prokaryotic origin that is concomitantly transferred to plants during transformation. To ensure public acceptance of the transfer of genes within species, gene transfer must be achieved without the presence of any other DNA from "foreign" sources.

Safe and sufficient food production is an important issue in India. Development of improved varieties by modern plant breeding is crucial, especially when global warming, population growth, environmental stresses, diminishing land resources associated with increased demand for quality food like healthy fruits and vegetables are considered as serious challenges in the future. To master these challenges, a second green revolution is needed in the country. (10)

During the first green revolution, India had taken the lead by translating new scientific developments like short straw traits. Transformation of locally adapted varieties into short straw varieties by backcrossing resulted in higher yields.(11)

Nowadays, GM varieties are emerging as a strong tool and promise. One of the major outcomes until now in GM technology is yield security by resistance against pathogens, pests and total herbicides**.**

These GM crops are at the moment connected with transgenes and worldwide. Biotech Crop hectares increased by an unprecedented 100-fold, from 1.7 million hectares in 1996, to 170 million hectares in 2012, (12**)** suggesting that crop biotechnology is one of the fastest adopted technologies in recent history. Recent major developments in science, like genome sequencing, are being applied in crop plants like rice (13), wheat (http://www.wheatgenome.org) maize and potato, and efficient gene isolation methods such as map-based cloning and allele mining have opened up new avenues in plant breeding using cloned indigenous genes. Natural indigenous genes, isolated from the crop plant itself or from crossable species, are now called cisgenes in order to distinguish them from transgenes. The traits of these cisgenes represent the existing genetic variation applied in classical plant breeding.

DEFINITIONS OF KEY TERMS IN RELATION TO PLANTS

Cisgenesis:

The term cisgenesis was coined by Schouten and colleagues (9). They claimed that – despite using the same genetic modification techniques as in transgenesis – "cisgenic" plants could be compared to traditionally bred plants as the concept involves only genes from the plant itself or from a close relative. These genes could also be transferred by traditional breeding methods. The genomic region containing the gene of interest is left contiguous, including all regulatory elements. Is the genetic modification of a recipient plant with a natural gene from a crossable—sexually compatible—plant. Such a gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation.Cisgenic plants can harbour one or more cisgenes, but they do not contain any transgenes.

Transgenesis :

Categorizing genetically modified (7) – or transgenic – organisms (*i.e.* organisms changed by receiving hereditary material from another organism) according to the origin of the genetic material used for the modification. Based on the "genetic relatedness between the donor and the recipient organisms", five categories of GMOs were presented. Is the genetic modification of a recipient plant with one or more genes from any non-plant organism, or from a donor plant that is sexually incompatible with the recipient plant? This includes gene sequences of any origin in the anti-sense orientation, any artificial combination of a coding sequence and a regulatory sequence, such as a promoter from another gene, or a synthetic gene.

Intragenes:

It composed of genetic elements originating from the crop species itself or from crossable plant species. Genetic elements are, e.g., promoters, coding regions, and DNA sequences that are similar to T-DNA borders from *Agrobacterium tumefaciens*. These elements can originate from different genes and loci. For example, promoters can be chosen to alter the expression of a native gene. Intragenes are used in vectors from which the sequences of the L- and R-borders are originating from plant-DNA. (14)

Traditional Plant Breeding:

The gene pool for the traditional plant breeder consists of genes from the species itself, from crossable wild species, and genes altered by induced or spontaneous mutations. Sexual compatibility in traditional plant breeding includes the application of embryo rescue, and crosses with bridging species. All these techniques result into non-GM varieties without the need of applying additional rules such as GMO regulations.

In potato, variety development is nowadays dependent on the selection of more than 100,000 seedlings per new variety (15). Main reason for that is, as indicated above, the growing number of traits that have to be combined into one genotype. Another reason is that the gene sources for important traits such as disease resistances or quality traits are frequently found in wild species. Introgression of such traits into a variety needs a lot of pre-breeding in which the gene of interest has to be “cleaned” from linkage drag caused by disturbing neighboring wild alleles. Stacking of several introgression traits from one or more wild species into a single variety worsens this problem. In cash crops such as tomato, but also in wheat, marker-assisted selection can help to speed up the process of combining several traits. In lettuce, an example has been described that marker-assisted selection can assist breeders in reducing linkage drag problem. However, marker-assisted selection is not easy. It still has to be further developed to allow for practical use in potato breeding and in breeding of many other crops.

In potato, traditional introgression breeding has been successful in breeding for resistance to viruses and nematodes but it failed for sustainable resistance to *P. infestans*. The utilized sources of resistance against *P. infestans* were mainly tracing back to *Solanum demissum*. For this species 11 R-genes against *P. infestans* have been described . All these resistance genes have been broken down and only four of them have been used extensively in varieties. Recently, in laboratory over 1,000 accessions of 200 different species have been screened for resistance and new sources of resistance have been found (16).

The most important new source in traditional resistance breeding until now is *S. bulbocastanum*. It contains several useful R-genes with broad spectrum resistance. Breeding with this source took more than 30 years before the first resistant varieties such as cv. Toluca was introduced into the market.. Main reason for that was the need for multiple bridge crosses with *S. acaule* and *S phureja* before the R-gene source came available into the potato background.

Bridge cross hybrids with distantly related species like *S. bulbocastanum* have the additional disadvantage that meiotic recombination is decreased, hampering removal of disturbing linkage drag. Variety development takes even much more time if stacking of several R-genes from such far related species is needed. If such valuable R-gene sources are used one by one in new varieties, the arms race against the pathogen will remain very difficult. There is a serious danger of burning down important resistance genes in potato one by one, if they are introduced as single genes. For stacking several effective resistance genes, molecular information is required in the selection process.

Another way of breeding is improving existing varieties. For vegetative, heterozygous crops, there are two ways of improvement of existing varieties with a long history of safe use, i.e. (induced) mutation breeding and genetic modification. Mutation breeding is still popular in vegetatively propagated ornamentals that are heterozygous like rose, *Alstroemeria, Chrysanthemum* but also in fruit trees like apple and peach. Main reason for improvement is not always the solution of problems but alteration of the phenotype with added value in the market. In potato, mutation breeding has been mainly restricted to altered traits like tuber skin colour and other tuber related traits. (17)

The second possibility for improving existing varieties is genetic modification. With a restricted number of large crops like soybean, maize and cotton experience has been obtained. In the near future not only transgenes but also cisgenes and intragenes will become available.

Evergreen Revolution
In 1990, I introduced the term Evergreen Revolution to emphasize the need for enhancing productivity in perpetuity without ecological harm. In population rich, but land-hungry countries like India, China, and Bangladesh, there is no option except to increase production under conditions of diminishing per capita availability of arable land and irrigation water and expanding biotic and a biotic stresses. Evergreen Revolution is another term for sustainable agriculture and is based on tools developed by blending traditional ecological prudence and frontier technologies. (18), supporting my concept of Evergreen Revolution.

The problem before us is how to feed billions of new mouths over the next several decades and save the rest of life at the same time, without being trapped in a Faustian bargain that threatens freedom and security. No one knows the exact solution to this dilemma. The benefit must come from an Evergreen Revolution. The aim of this new thrust is to lift food production well above the level obtained by the Green Revolution of the 1960s, using technology and regulatory policy more advanced and even safer than those now in existence.

Irrigation water will be a serious constraint in the coming decades. Similarly, land degradation threatens both sustainable agriculture and food security. Both soil restoration and enhancement, and water conservation and sustainable use are important for launching an Evergreen Revolution movement. It would be useful to consider recent advances in the improvement of wheat and rice to examine what midcourse corrections are needed for the purpose of adding the environmental dimension to productivity improvement.

Paradigm shift in research strategy

It is now widely agreed that new technologies must be not only economically viable, but also environmentally and socially sustainable. The term ecotechnology is used in the case of technologies that are rooted in the principles of ecology, economics, gender and social equity, employment generation, and energy conservation. Ecotechnologies will require an interdisciplinary approach and will need research based on an entire farming system. Thus, there has to be a paradigm shift in research strategies from a commodity-centered approach to an integrated natural resources management procedure covering the entire cropping system. Crop–livestock integrated farming is particularly important for soil fertility buildup and for multiple sources of income. (19).

Second green revolution in traditional breeding by cisgenesis

Cisgenesis is better than traditional introgression and translocation breeding because of the absence of linkage drag and the reduced number of steps. Domestication of crop plants has diminished the presence of toxic components into acceptable levels, and there is increased possibility of acquiring unrelated traits/toxic compounds when wild species are used for crop improvement. In cisgenesis these problems can be eliminated easily and desired traits can be incorporated by gene stacking.

For example, cisgenic apple varieties are being evolved by stacking resistance genes from crossable *Malus* plants for durable résistance to apple scab, and biotic interaction between potato–*Phytophthora infestans* is being studied for durable resistance by stacking several *R*-genes. Similar approach can be attempted for developing cisgenic crop plants for quality traits and biotic/a biotic stress tolerance in India.

Cisgenesis can be applied for all dominantly inherited traits. A second green revolution is needed in India to overcome the challenges related to yield security, quality traits and healthy vegetables and fruits. Integrated gene management along with precision farming is suggested for evergreen agricultural revolution1. We believe cisgenesis can open up new options for evergreen revolution. For many crops, including vegetatively propagated ones, cisgenesis can be used directly for the improvement of existing varieties, which have already been shown to be safe for use in the market.

Cisgenecis are different from traditional breeding

1). The donor sequence is inserted into the genome at an *a priori* unknown position, which might affect DNA methylation and other factors that in turn can influence gene expression. A biological counterargument is that translocations and (de)methylations also occur in nature. (20) showed that Helitron transposons in maize capture a 5.9 kilobaselong DNA fragment containing three genes and move it to another part of the maize genome.

2). The insertion of a cisgene results in a mutation at the insertion site. Moreover, rearrangements or translocations might occur in the flanking regions. These mutations might knock out genes, open new reading frames and thereby induce phenotypic effects. But natural mutations and rearrangements in plant genomes are common, especially in chromosome regions where transposons are active. Unintended genome reorganization can also be induced by pathogen attack, a biotic stress and interspecies hybridization. A regulatory counterargument is that, in Europe, mutation breeding is now exempt from the regulations on the release of GMOs into the environment.

3). The donor sequence does not replace an allelic sequence, but is added to the recipient species' genome. Owing to the process of gene transfer, it is possible that the new sequence is inserted several times in one genome, which might affect gene expression and, therefore, phenotype. However, gene duplication is a common natural occurrence, for instance in the case of resistance genes or other multigene families.

4). The cisgenic plant might contain some small, non-coding sequences from the vector such as T-DNA borders, which are 25-base-pair imperfect repeats that delimit the DNA segment transferred to plant cells when using *Agrobacterium* mediated gene transfer. Other non-coding sequences from the vector might be parts of a multiple cloning site or remnants from recombination sites that were used to excise undesired DNA sequences, such as a selection gene, after the DNA transfer.

Difference between transgenesis and cisgenecis
Transgenesis
✓ In the case of transgenesis, the transferred gene usually derives from an alien species that is neither the recipient species nor a close, sexually compatible relative.
✓ Transgenesis can extend the gene pool of the recipient species. Such a novel gene might provide the target plant with a new trait that neither occurs in the recipient species in nature nor can be introduced through traditional breeding.
✓ This novel trait might affect the fitness of the recipient species in various ways; a change in fitness can then spread through gene flow between a GM crop and its wild relatives, potentially creating shifts in natural vegetation.
✓ Consequently, lawmakers and regulatory authorities have paid much attention to the safety of deliberate releases of transgenic crops into the environment and have put in place biosafety frameworks to control this risk.(21)

Cisgenecis
✓ In the case of a cisgenic plant, the gene of interest, together with its promoter, has been present in the species or in a sexually compatible relative for centuries.
✓ Therefore cisgenesis does not alter the gene pool of the recipient species and provides no additional traits.
✓ No changes in fitness occur that would not happen through either traditional breeding or natural gene flow. Similarly, cisgenesis carries no risks—such as effects on nontarget organisms or soil ecosystems, toxicity or a possible allergy risk for GM food or feed—other than those that are also incurred by traditional breeding. This is the fundamental difference between cisgenesis and transgenesis.
✓ Consequently, the deliberate release and market introduction of cisgenic plants is as safe as the release and market introduction of traditionally bred plants. On the issue of safety, regulators could treat cisgenic plants the same as conventionally bred plants.

Cisgenesis and Food Safety
For these crops agriculturally important cisgenes are becoming more and more available from specific research programmes but also from sequencing of whole genomes. In other words, the almost ideal way of "clean" introgression breeding with insertion of only the target gene is feasible now. Instead of intensive pre-breeding for introgression of alien target genes and removal of many disturbing neighboring alien alleles from the wild breeding parent, a targeted gene cloning strategy followed by transformation can be developed for isolating the useful alleles coding for, e.g., broad spectrum disease resistances or for quality traits such as yellow flesh in potato and red flesh in apple. The yellow flesh *Y* gene itself or the *Or* allele present in *S. phureja* for orange flesh colour are available for improving existing white fleshed varieties by metabolic engineering which is of importance in poor areas in order to improve vitamin A content, as earlier described for golden rice

1). The apple scab resistance project is isolating different resistance genes, and introduces these natural apple genes into existing elite varieties for obtaining durable polygenic resistance. The new cisgenic approach does not need introgression breeding and stacking of resistance genes by expensive pre-breeding with wild material. However, resistance breeding using cisgenesis is the new pre-breeding approach, starting with isolation of new natural, broad spectrum, resistance genes, followed by direct, stacked, introduction via cisgenesis into existing elite varieties. Also the *MdMYB10* gene for red fleshed apples is being introduced into elite cultivars, enhancing the anti-oxidant capacity of the apples strongly. (22)

2). Proof of principle in breeding for durable resistance of potato to *P. infestans*. , the societal costs and the cisgenic approach have been described. It is based on the isolation of different classes of broad spectrum R-genes recognized by the use of isolated Avr-genes, present in the different isolates of this pathogen, and the combined application of the R-genes in existing and new varieties.. The isolation of several broad spectrum R-genes with their cross reacting Avr-genes is enabling testing of their individual biological function after stacking of them by cisgenesis or even by introgression breeding. Stacking of broad spectrum R-genes by cisgenes in existing varieties with a history of safe use is an additional strong recommendation. These improved varieties also can be used as breeding parents for developing new varieties **(**2).

3) *O. glaberrima* (23*)* is a cultivated rice species endemic to Africa. Two major ecotypes are recognized in this species – floating photosensitive ecotype grown in deep water, including coastal mangrove areas, and an early erect ecotype grown in upland or moderately inundated lowlands. The Asian rice species *O. sativa* (genome AA, 2*n* = 24) is spread in large parts of the world and is more diverse than *O. glaberrima*. *O. sativa* is broadly divided into *indica* and *japonica* subspecies. *O. glaberrima* originated around 1500 BC in swampy basins of the upper river delta of Niger in West Africa, which is the primary centre of origin of this species. Recently, *O. glaberrima* genome has been used as a reference to unravel the sequence of evolution in *O. sativa* subspecies *O. glaberrima* differs from *O. sativa* in many qualitative and quantitative traits. The two species can be distinguished in the field by differences in ligule shape and panicle branching.

THE TORETICAL DRAWBACKS

1) Unknown insertion site:

A frequently mentioned drawback is the fact that cisgenes will be inserted at unknown places of the plant genome, which could bring unforeseen risks.

2) Mutation at insertion site:

Another theoretical drawback that is mentioned regarding cisgenesis is the mutation made at the insertion site of the cisgene and the unexpected accompanying phenotypic changes that might result from this. We just have seen that induced translocations and especially naturally occurring transposons can cause mutations in the crop genome, which even can be used. Usually, mutations are inherited recessively and represent loss of function.

3) Food safety:

An important advantage of cisgenesis is food safety. The use of wild *Solanum* species as source of genetic variation is bringing back different kinds of glycoalkaloids that have been removed during the breeding process in the past. The use of wild species as new source of genetic variation is accompanied by the re-introduction of these compounds.

Objections against exemption and clarification:

A few objections against cisgenesis to exempt them from the GM-regulation are:
(1) The random insertion of the gene into the genome.
(2) Mutation caused in the plant genome.

In plant breeding such events are not new. We know from induced translocation breeding by irradiation that a piece of alien chromosome containing the desired resistance trait of the wild species, surrounded by many other donor genes, is inserted randomly in the crop genome, and such an approach is employed in wheat. In crops like maize, transposable elements can move within the genome. In practice induced mutation breeding is safe, as illustrated by safe use of more than 2500 mutant varieties in many different crops. The new GM varieties with transgenes are good problems, if selection procedures of normal breeding are applied.

CONCLUSION

The classical methods of alien gene transfer by traditional breeding yielded fruitful results. However, modern varieties demand a growing number of combined traits, for which pre-breeding methods with wild species are often needed. Introgression and translocation breeding require time-consuming backcrosses and simultaneous selection steps to overcome linkage drag. Breeding of crops using the traditional sources of genetic variation by cisgenesis can speed up the whole process dramatically, along with usage of existing promising varieties. This is specifically the case with complex (allo) polyploids and with heterozygous, vegetative propagated crops. Therefore, we believe that cisgenesis is the basis of the second/ever green revolution needed in traditional plant breeding.

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