Chemical Science Review and Letters ISSN 2278-6783

Review Article

The Cisgenesis New Tool of Breeding: A Review

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Abstract

Biotechnology as such could not be exploited to its full potential because of several reasons, the major one being its acceptance by the consumers due to biosafety issues. Scientists have been continuously looking for its alternatives where biosafety issues do not exist. Cisgenesis is one such approach. It is the genetic modification of a recipient plant with a natural gene from a crossable-sexually compatible-plant. Cisgenesis products are similar to the products of traditional breeding which encompasses all plant breeding methods where gene transfer is carried out among sexually compatible species. On the issue of safety regulators cisgenic plants could treat same as conventionally bred plants. Indeed, cisgenesis has great potential to overcome a major bottleneck in traditional breeding. During introgression breeding, a wild plant with an interesting trait is crossed with a high-quality genotype, such as a cultivar. The wild plant, however, passes on not only its genes of interest to the progeny but also other, sometimes deleterious, genes. This so-called linkage drag can slow down the breeding process tremendously, especially if the gene of interest is genetically tightly linked to one or more deleterious genes. By contrast, cisgenesis isolates only the gene of interest from the donor plant, which is then inserted into the recipient in one step. As no other genes are transferred, this method avoids linkage drag. Cisgenesis is one step transfer of genes instead of 6-8 generations required by the conventional breeding methods. Cisgenesis is a efficient method for cross-fertilizing heterozygous plants that propagate vegetatively, such as banana, potato and apple. It can directly improve an existing variety without disturbing the genetic make-up of the plant. The cisgenesis plays important role in resistance breeding, the introduction of the apple scab resistance gene *Vf* from a wild into a cultivated apple, which began as early as the 1950s. Similarly, breeding programmes aimed at rendering potatoes with durable resistance to the potato-late-blight-causing oomycete *Phytophthora infestans* require a series of genes from resistant wild species such as *Solanum demissum* and *S. bulbocastanum*.

Keywords: Agronomic traits, variability and mean of parameter

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Introduction

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.

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. Plant genetic resources represent valuable sources of genetic variability for crop breeding. The development of novel biotechnologies is necessary for increasing the efficiency of their use in pre-breeding and breeding work. The genome sequencing of hundreds of genotypes and the mining of allele diversity in major crops and populations of landraces and wild relatives allow the isolation of genes underlying characters of interest and their precise modification or transfer into targeted varieties. The technological developments and applications of new plant breeding techniques (NPBT) that maximize the similarity with gene transfer by crossing (cisgenesis/intragenesis) or the accuracy of biotechnological approaches (genome editing) are reviewed. Their potentialities and current limitations as well as the possible advantages of using them separately or combined for the exploitation of PGR in crop breeding are also discussed. Above-mentioned NPBT tackle some objections to the application of biotechnologies in agriculture and are under review worldwide to assess the possible exclusion from the current regulation systems for genetically modified plants.

Plant genetic resources (PGR), including landraces (LR), heirloom varieties (HV) and crop wild relatives (CWR), are paramount important sources of genetic variability at the intraspecific and interspecific level for breeding new crop varieties. Due to their history, based on natural and artificial (farmer) selection in particular environments, LRs are adapted to local growing conditions and show interesting features in terms of resilience and organoleptic and nutritional value [1, 2]. Besides their indirect use as a source of genes for the development of new varieties, they can be also used directly by farmers. Nevertheless, they often show some specific defects, for example susceptibility to one or more pathogen strains, that limit their use. Although LRs and CWRs are being used in intraspecific/interspecific hybridizations and introgression breeding for long time [3-5], the development of novel technologies is necessary for increasing the efficiency of their use in pre-breeding and breeding work, in order to promptly respond to present and future agricultural challenges.

At the end of the last century, genetic engineering techniques enabled the production of the first generation of genetically modified plants (GMP or transgenic plants), based on the transfer and random insertion into the host plant genomes of genes isolated from other plant species or from other organisms. In this century, the improvement of sequencing technologies has allowed the complete deciphering of genomes in many species of agricultural interest, such as rice, maize, tomato, potato, pepper, eggplant, cucumber, melon and others [6-8]. Next-generation sequencing (NGS) technologies consent the whole or targeted genome resequencing of hundreds of genotypes and the mining of allele diversity in the populations of crop landraces and wild relatives [9-13]. Knowledge of the structure and function of plant genomes in major agricultural and related PGR prompts the development of so-called second-generation biotechnologies and their application in breeding (new plant breeding techniques or NPBT), aiming to the isolation of genes underlying the characters of interest and their precise modification or transfer into targeted varieties [14-17].

In relation to the use of transgenic plants in agriculture, the random insertion of the transgene into the genome of crop plants are sometimes perceived as potential risks to the environment and health. NPBT that maximize the similarity with gene transfer by crossing (cisgenesis/intragenesis) or the accuracy of biotechnological approaches (genome editing) tackle some objections to the application of biotechnologies in agriculture. They are under review worldwide to assess the possible exclusion from the current GMP regulation systems [14, 18-20]. Hereinafter, the recent developments of cisgenic/intragenic and genome editing approaches based on the advancement of sequencing technologies and the availability of genome information are reviewed, evaluating their potentialities and current limitations as well as the possible advantages of using them separately or combined for the exploitation of PGR in crop breeding.

Cisgenesis/Intragenesis

Although several variations have been proposed [21], both cisgenic and intragenic approaches are based on the transfer of only genes and regulatory sequences derived from other genotypes of the same or sexually compatible species. In case of cisgenesis, the entire gene with its own regulatory sequences and in sense orientation is transferred. In the second case (intragenesis), different coding and regulatory sequences are assembled either in sense or in antisense orientation, the latter if the aim is to reduce gene expression by activating the RNA interference (RNAi) pathway. Further, according to some authors, P-DNA borders and vector backbone sequences derived from the sexually compatible gene pool should be used in intragenic vectors for *Agrobacterium*-mediated transformation [22]. While, at least in principle, cisgenic products can be achieved by conventional breeding, the same is not true for intragenic ones. Anyway, in both approaches, only the genes that code for the characters to be modified should be eventually present in regenerated plants and a number of technologies can be adopted to avoid the presence of

selectable marker genes generally used for *in vitro* selection of transformed cells [23]. Conventional gene delivery methods, such as either *Agrobacterium* spp. or biolistics-based transformation, are commonly used for transformation.

Resistance Breeding

As recently reviewed [24, 25], the cisgenic approach has been used to improve pathogen resistance and quality traits in several crops, namely durum wheat, poplar, grape, apple, potato. Approaches aimed at increasing intragenic gene expression have been published in apple, strawberry and potato to increase the resistance to pathogens and in perennial ryegrass tolerance to drought, whereas the targeted reduction in gene expression was obtained in potato and alfalfa with the aim to improve various aspects of quality (**Table 1**). Not all products so far classified as intragenic or cisgenic, however, fully comply with requirements set for such kinds of plants, because some still contain microbial regulatory sequences and/or selectable marker genes [23]. More recently, molecular characterization of true cisgenic apple plants previously produced and expressing the*Rvi6* scab resistance genes has been reported in detail [26], while new cisgenic apples with the same trait have been developed using an alternative recombinase system [27]. Another example is also of a cisgenic apple line C44.4.146 which was regenerated using the cisgene *FB_MR5* from wild apple *Malus* × *robusta* 5 (*Mr*5), and the previously established method involving *Agrobacterium tumefaciens*-mediated transformation of the fire blight susceptible cultivar 'Gala Galaxy' using the binary vector p9-Dao-FLPi. The line C44.4.146 was shown to carry only the cisgene *FB*_MR5 [28].

Furthermore, marker-free cisgenic potato (*Solanum tuberosum*) plants expressing late blight resistance genes from *S. stoloniferum* (*Rpi-sto1*) and *S. venturii* (*Rpi-vnt1.1*) have been produced by *Agrobacterium*-mediated transformation without using any marker gene but PCR for the selection of transformed plants. Due to the activity of both introduced *R* genes, cisgenic plants showing broad-spectrum late blight resistance could be selected [29]. An intragenic vector for future applications has been developed in *Citrus* spp. [30].

Table 1. Examples of agronomic traits modified through the application of either *cisgenesis* or *intragenesis* in various crops

Species	Trait	Gene	Technology	References
Alfalfa	Lignin content	*Comt*	Intragenesis	[31]
Apple	Scab resistance	*Rvi6* (*HcrVf2*)	Cisgenesis, intragenesis	[32]; [26]; [33]
Barley	Grain phytase activity	*HvPAPhy_a*	Cisgenesis	[21]
Durum wheat	Baking quality	*1Dy10*	Cisgenesis	[34]
Perennial ryegrass	Drought tolerance	*Lpvp1*	Intragenesis	[35]
Poplar	Plant growth and stature, wood properties	*PtGA20ox7, PtGA2ox2, PtRGL1_1, PtRGL1_2, PtGAI1*	Cisgenesis	[36]
Potato	Late blight resistance	*Rpi-sto1*, *Rpi-vnt1.1*	Cisgenesis	[29]
	High amylopectin	*GBSS*	Intragenesis	[37]
	Prevention of black spot bruise	*Ppo*	Intragenesis	[38]
	Accumulation of reducing sugars after cold storage and acrylamide after high-temperature processing	*R1, PhL, StAs1, StAS2*	Intragenesis	[39]; [40];[41]
Strawberry	Grey mould resistance	*PGIP*	Intragenesis	[42]

Because, the sequences transferred by either cisgenesis or intragenesis are derived from the same or related species, the knowledge of their sequence, position and function in the genomes of origin is essential. It is expected that such knowledge will prompt a wider use of both technologies in place of common transgenesis. Although gene transfer within the same or evolutionarily close species can also be achieved by conventional breeding, cisgenic/intragenic approaches reduce considerably both the duration of selection steps and linkage drag. In addition, the genotype and phenotype of varieties to be modified remain largely unchanged, an issue particularly important with vegetatively propagated heterozygous and/or long-cycle species, such as potato or, in general, fruit trees.

Another potential advantage of cisgenesis/intragenesis, compared to conventional breeding, is the higher knowledge of transferred sequences [22].

Regulation of Cisgenics

The EFSA (European Food Safety Authority) concluded that cisgenic plants pose risks similar to those obtained with conventional breeding, while the risks of plants produced by intragenesis are similar to those of transgenic plants (EFSA Panel on Genetically Modified Organisms [43]. Furthermore, several recent reports confirmed a greater acceptance by consumers of cisgenic products compared to the corresponding transgenics [44]. As far as the regulation of these products is concerned, generally speaking, United States, Canada and Australia are showing a more open orientation and are aiming to distinguish them from conventional GMPs [16, 18], while in Europe and other countries, the attitude is more cautious.

Notwithstanding the aforementioned considerations, cisgenic/intragenic approaches still show some drawbacks that limit a wider application. A careful analysis of regulatory sequence type and size used in cisgenic/intragenic constructs is necessary. In apple, although an acceptable level of scab resistance was achieved, differences in expression level of the *Rvi6* gene depended on using the *rbcS* promoter from the same species or the native promoter with different lengths [26, 32, 33]. Additional effects of the terminator type and length were discussed by the same authors. Variability in gene expression could be also due to random insertion of the cisgene in the host genome, determining negative position-dependent epigenetic regulation [26]. Further, random integration of cisgenes/ intragenes can potentially determine, similar to regular transgenes, the interruption and silencing of resident genes or other relevant sequences and so a thorough screening of regenerated plants is necessary. Finally, the number of gene copies and the amount of vector backbone sequences transferred is an additional issue, because up to 80% of original plants regenerated from cisgenic transformation experiments showed the integration of vector backbone sequences [26, 29]. New vectors containing buffer plant sequences or genes for counter selection in the backbone can help limit the negative consequences of unprecise integration of cisgenes and intragenes in the host genome. Furthermore, vectors composed of only plant-derived sequences have been proposed. The use of minimal linear cassettes and biolistic gene delivery is also a valid method to have 'cleaner' transformations [22, 30, 34].

Genome Editing

'Genome editing' includes a set of techniques that allow to edit, delete, replace or insert, in a targeted site, specific genomic sequences of interest. They are based on the induction, in different organisms, including plants, of cuts in double-strand DNA (DSB, double-strand breaks), which are then 'repaired' with two different processes: the non-homologous end-joining (NHEJ) or homology-directed repair (HDR) [45-48].

The breaks in the double-strand DNA can be induced by four systems based on specific enzymes: (i) Meganucleases, (ii) Zinc finger nucleases (ZFN), (iii) Transcription activator-like effector nucleases (TALEN) and (iv) Clustered regular interspaced short palindromic repeats/CRISPR-associated nucleases (CRISPR/Cas). In comparison with the meganucleases, the other systems allow to edit more efficiently 'target' sequences and hence have a larger use than the former for different purposes. ZFN and TALEN are based on the association between some proteins, which have the property to recognize and bind in a selective manner to preselected nucleotide sequences, and two units of a nuclease (*Fok*I), which, when dimerized, cut double-strand DNA. In order to obtain dimerization of *Fok*I, a couple of ZF or TALE proteins are designed and synthesized according to the nucleotide sequence to be modified. Each ZF protein recognizes three nucleotides on each DNA strand, and generally, each ZFN uses 3–4 proteins per filament: in this way, the specificity is determined by 18–24 nucleotides (9–12 per filament). In the TALEN system, the specific recognition with the DNA sequence is determined by the TALE proteins (discovered in phytopathogenic *Xanthomonas* spp. bacteria), each of which recognizes a single nucleotide. Specific recognition of the region to be modified is determined by thirty-forty repeats (15–20 per filament), each containing 33–35 amino acids.

The sequence of each repeat is highly conserved, except at positions 12 and 13 (RVDs, repeat variable diresidues). Each repeat recognizes a single base. The dimeric *Fok*I cuts the DNA between the 'batteries' of ZF or TALE proteins. Unlike ZFN and TALEN, in CRISPR/Cas9 technology, recognition of the DNA sequence to be modified is determined not by proteins, but by a chimeric sequence of RNA (single guide RNA – sgRNA), which results from the fusion of the two sequences (crRNA and tracrRNA) present in the natural system (the CRISPR/Cas

system is used by bacteria to defend themselves against phages), while the cutting of DNA sequence is performed by the associated monomeric enzyme Cas9 [49-53].

After the two strands of the double helix of DNA are cut, in the absence of foreign donor sequences, the filaments are predominantly rejoined in plants by NHEJ, but, because this process is susceptible to errors, small changes (mostly frame-shift mutations due to insertions and deletions) in the original sequence are induced, which generally result in the loss of function of the target gene and a mutated phenotype. This result is similar to that obtainable with other technologies in use since long time, such as mutagenesis with chemical or physical mutagens, although the induction of mutations is not random in the genome, as in classical mutagenesis, but limited to genes of interest. As a consequence of conventional random mutagenesis, unwanted mutations can be induced throughout the genome and large-scale screens of mutagenized populations are needed to identify those plants with mutations of interest. Antisense and RNAig-based technologies can be also used to selectively knockdown gene expression. Their effects, however, are often incomplete, not stable across generations and not limited to the gene of interest. In addition, they target transcripts, but no other genetic elements, such as promoters, enhancers, introns and intergenic regions, which are instead accessible by genome editing tools. If, together with the nucleases described above, appropriate DNA fragments homologous to the target sequence are also inserted into the cell, they can, using the mechanism of homologous recombination (HDR), which unlike NHEJ does not induce errors, replace (correct) some nucleotide sequences of the gene to be modified or add new genes or regulatory elements in a predetermined position of the genome [54]. The induction of site-specific random mutations (1), the induction of mutations in a predefined sequence of a particular gene (2) and the replacement or the insertion of an entire gene (3) are collectively dubbed as SDN (site-directed nuclease)-1, SDN-2 and SDN-3, respectively (EFSA Panel on Genetically Modified Organisms [55].

Delivery into plant cells of various components necessary for genome editing has been accomplished by either transient or stable transformation methods, including protoplast systems, *Agrobacterium*-based procedures, biolistics and virus vectors. Transient methods are preferable both for the cytotoxicity sometimes shown when nucleases are expressed stably and for regulatory reasons, in order to avoid the presence of stably integrated exogenous sequences. The choice of one method or another, however, depends on several factors, such as the length of life cycle, the feasibility of protoplast isolation and plant regeneration, the type of molecule to be expressed, the repairing mechanism and the editing objective pursued [48, 56-63].

The three main methods presently available for genome editing in plants have been recently compared for several aspects in various reviews [54, 59, 60, 61, 62, 64, 65]. In comparison with ZFNs and TALENs, the CRISPR/Cas approach looks more attractive based on higher simplicity, accessibility, cost, versatility, possibility of multiplexing and other aspects, including an easier open access to resources. Although contrasting results have been reported, the off-target effects remain the main concern for a wider application of genome editing to functional genomics and plant breeding.

Bioinformatic analysis of target genomes is necessary to avoid targeting sequences repeated throughout the genome, and to this end, several tools available online are being developed. Furthermore, the double nickase and Cas9-nuclease fusion systems were developed to reduce off-target editing of CRISPR/Cas9 [59, 63]. The relatively large size of Cas9 enzyme can be a limiting factor in some cases, for example when it has to be delivered through viral vectors, whose cell-to-cell movement necessary to*in vivo* edit meristematic or gametic cells is prevented by their genome size [63].

Anyway, a novel smaller version of Cas9 isolated from *Staphylococcus aureus* edited the mouse genome with efficiencies similar to those of the widely used form from *Streptococcus pyogenes* [66]. Additional future technological improvements will derive by the recent discovery in diverse bacteria of the single RNA-guided endonuclease Cpf1. In comparison with Cas9, it shows distinct advantages, including the production of sticky ends after cutting DNA instead of blunt ends, a feature that should make editing by sequence insertion/replacement easier and more controllable [67].

Due to difficulties in transferring in plant cells a DNA repair template together with nucleases, and to the intrinsic low efficiency of homologous recombination in higher plant nuclear genomes, most reports deal with targeted mutagenesis (gene-knockout or SDN-1 approaches). As far as crop plants are concerned, mutations in targeted genes were obtained for quality-related and pathogen or herbicide resistance traits in cereals (barley, maize, rice, sorghum and wheat) [68-78], soybean [79-83], potato [84-86], tomato [87, 88] tobacco [89] and perennial fruit trees (poplar, sweet orange, apple and fig) [90-93], although in some cases mutations were only induced in reporter genes and/or studied *in vitro* at the molecular level. Some examples in which traits of agronomic interest were investigated in full plants are summarized in **Table 2**. Interestingly, they include worldwide important crops, such as potato, soybean,

rice and wheat. In the latter, by TALEN, it was possible to induce mutations in all three *TaMLO* (mildew-resistance locus) homoeologs, thus conferring resistance to *Blumeria graminis* f. sp. *tritici*, a trait not found in natural populations. HDR-mediated approaches including gene editing (SDN-2) or gene replacement/stacking (SDN-3) were achieved in tobacco, soybean, barley, rice and maize crops, besides model *A. thaliana* and *Nicotiana benthamiana*. In tobacco, mutations known to confer herbicide resistance were introduced into *SuR* genes by ZFN, confirming the acquisition of resistance in calli [94], and by TALEN [95]. *ALS* genes, conferring herbicide resistance, were successfully edited also in maize and soybean by CRISPR/Cas9 [82, 74]. In barley, a single amino acid exchange inducing GFP to YFP conversion was achieved by TALEN-induced modification of a*gfp* transgene [96], while preselected sequence changes were induced in rice *PDS*-and *EPSPS*-coding genes by CISPR/Cas and TALEN systems, respectively [97, 98]. In maize, using the ZFN technology, the endogenous *IPK1* gene, involved in phytate accumulation, was disrupted by the insertion of the *PAT* gene, encoding a phosphinothricin acetyltransferase, achieving in modified plants, at the same time, herbicide tolerance and low accumulation of phytate [99], while, more recently, the *PAT* gene was precisely inserted at a preselected locus by HDR mediated by CRISPR/Cas9 [74]. These studies showed that with the use of TALEN and CRISPR, the frequency of gene targeting by HDR is higher and now practicable for crop breeding.

Table 2 Examples of agronomic traits modified through the application of *genome editing* approaches in various crops

Crop	Trait	Gene	Technology	References
1. a SDN, site-directed nuclease.				
2. b Tested *in vitro*.				
Gene knockout (SDN-1)[a]				
Rice	Resistance to bacterial blight	*OsSWEET14*;*OsSWEET13*	TALEN; CRISPR/Cas9	[71]; [100]
	Fragrance	*OsBADH2*	TALEN	[73]
Bread wheat	Resistance to powdery mildew	*TaMLO-A1, TaMLO-B1, TaMLO-D1*	TALEN	[76]
Maize	Phytate biosynthesis	*IPK1*	ZFN	[99]
	Leaf epicuticular wax composition	*glossy2 (gl2)*	TALEN	[68]
	Leaf development; Male fertility; Herbicide resistance	*LIG1; Ms26, Ms45; ALS1, ALS2*	CRISPR/Cas9	[74]
Soybean	Profile and unsaturation level of seed fatty acids	*FAD2-1A* and*FAD2-1B*	TALEN	[80]
Poplar	Lignin content; Condensed tannin content	*4CL1*; *4CL2*	CRISPR/Cas9	[93]
Potato	Accumulation of reducing sugars after cold storage and acrylamide after high-temperature processing	*VInv*	TALEN	[84]
	Accumulation of steroidal glycoalkaloids	*StSSR2*	TALEN	[101]
Tomato	Plant development	*PROCERA (PRO)*	TALEN	[88]
	Leaf development	*ARGONAUTE7 (SlAGO7)*	CRISPR/Cas9	[87]
Gene editing (SDN-2)				
Maize	Herbicide resistance	*ALS2*	CRISPR/Cas9	[74]
Soybean	Herbicide resistance	*ALS1*	CRISPR/Cas9	[82]
Tobacco	Herbicide resistance[b]	*ALS SuRA* and *SuRB*	ZFN	[94]
Gene replacement/stacking (SDN-3)				
Maize	Phytate production/herbicide resistance	*IPK1/PAT*	ZFN	[99]
	Herbicide resistanceb	*PAT*	CRISPR/Cas9	[74]

Provided that sequences encoding nucleases and other components are not present in the final edited products, plants modified by either SDN-1 or SDN-2 approaches are indistinguishable from similar plants obtained by conventional mutagenesis (although background mutations are by far less) or natural variation. In addition, it is difficult to evidence the modification occurred in a few nucleotides and thus distinguish them from unmodified original genotypes. Hence, many argue that such plants should not be considered transgenic and thus they should fall outside the boundaries of GMP regulation [47, 54], 58, 60, 61, 102]. Recent results about the possibility to carry out DNA-free editing of plant genomes with preassembled CRISPR-Cas9 ribonucleoproteins could contribute to alleviate regulatory concerns towards NPBT [103]. The US Ministry of Agriculture (USDA) recently declared that the products derived from genome editing approaches will be evaluated case by case, deregulating then corn plants with low phytate obtained by the use of ZFN-3 [104-106].

The European Commission has not yet delivered its opinion. Only about the SDN-3 approaches, EFSA concluded that, similar to conventional transgenic approaches, risks are related to the sequence of transferred genes, although there are less risks associated with the potential disruption of either coding or regulatory sequences following the transgene insertion (EFSA Panel on Genetically Modified Organisms (GMO), [107]. According to the same source, a reduced amount of specific data should be asked in some cases, for example, after site-specific transfer of a cisgene. Novel regulatory schemes for products obtained by different genome editing approaches have been recently proposed [108, 109].

Discussion and Perspectives

It is estimated that 7–15 million euro and 4–6 years are needed to obtain the authorization for the cultivation of GMP in the environment, an investment that only large multinational companies can bear [104,106, 110]. Although in 2014 the use of GMP in agriculture reached 181.5 million hectares in the world [111] and quite a number of edible crops showing biotic or abiotic stress tolerance and improved nutritional value are in the pipeline [112], the scale of investment for their approval has limited the number to a few crops (soybean, maize, cotton, canola) and a few characters (almost exclusively resistance to insects and herbicides).

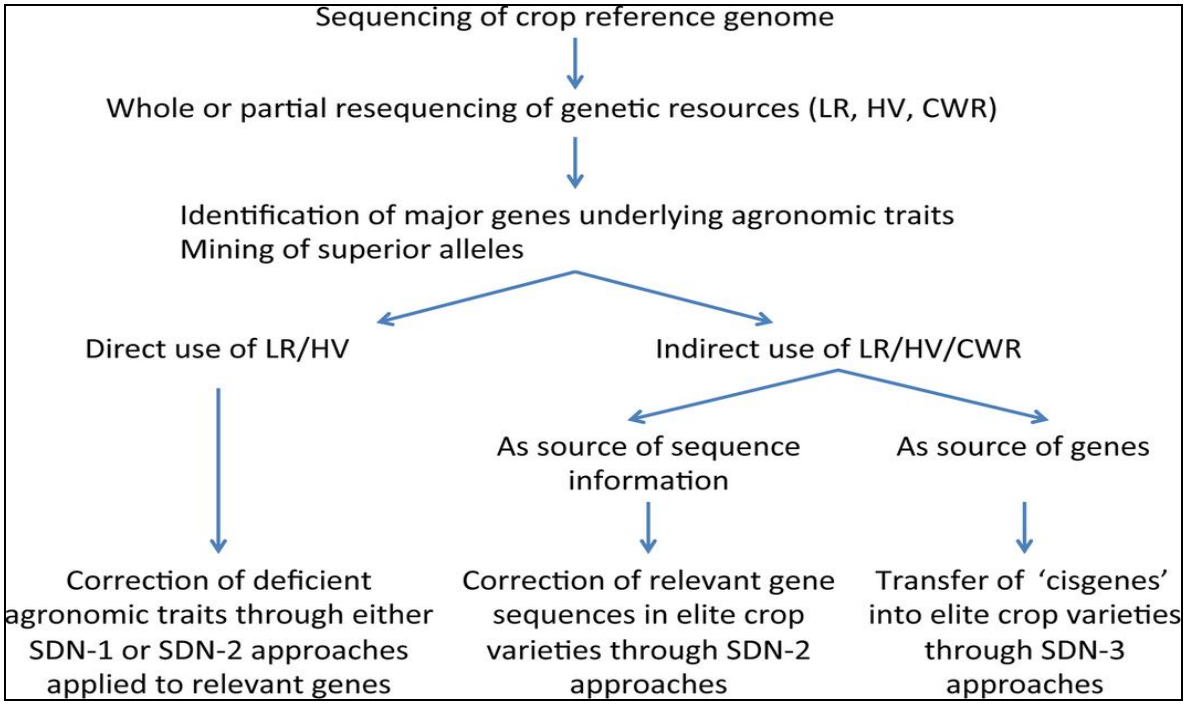

Figure 1: Implementation of genome sequence information and novel biotechnological approaches for the exploitation of genetic resources in crop breeding. LR: landraces; HV: heirloom varieties; CWR: crop wild relatives; SDN-1, 2, 3: site-directed nuclease usage for gene knockout, gene editing or gene replacement/stacking, respectively[23, 44, 113].

A more widespread implementation of novel biotechnologies in the characterization and exploitation of plant genetic resources for crop breeding is desirable. The implementation of NPBT in breeding and organic farming has been recently advocated in order to reintroduce in crop varieties the properties present in wild relatives ('rewilding') and close the productivity gap between organic and conventional agriculture [3, 5]. NPBT can be used in functional and complementation studies with the aim to identify major genes and superior mutations/alleles present in PGR (**Figure 1**). Besides agronomic aspects, the direct use of landraces and traditional varieties is appreciated for their 'cultural' value and their link to local 'traditional' agriculture. For a number of reasons, there is a general willingness to not modify their original genotype and phenotype. Hence, SDN-1 and SDN-2 approaches, based on the use of various nucleases, are the most convenient methods to precisely correct their defects, either inhibiting the expression of specific genes or enabling novel limiting features, leaving the genetic background and other traits largely untouched. For indirect uses of landraces, traditional varieties and crop wild relatives in plant breeding, structural and functional information derived from genomic studies in collections of such PGR can be adopted to edit gene sequences in elite crop varieties, targeting specific sites and inducing relevant mutations. Finally, PGR belonging to the 'breeders' genepool can be used also as source of cisgenes to be transferred in crop varieties, replacing alleles with superior counterparts or introducing new genes in the cultivated genepool. Although no scientific evidence has been related so far to a safer use of cisgenes instead of transgenes, a positive consumers' perception linked to the use of genes from compatible gene pools has been reported [23, 44, 113]. Nevertheless, the unpredictability of cisgene/intragene integration and expression is not only a limitation from the technical point of view, but also for public acceptance and regulatory issues [114].

Hence, the combination of cisgene/intragene concepts with those of new genome editing techniques can help reduce the concerns associated with the use of biotechnologies in agriculture, opening new perspectives for a more efficient use of genetic resources in crop breeding, valorizing the genomic and phenomic data that national and international initiatives are producing (http://www.divseek.org/). Further, editing of genes coding for proteins involved in chromosome segregation (e.g. kinetochore protein CENH3) could facilitate the production of haploids and the fixation of useful traits derived by introgression breeding in crops where efficient methods for haploidization are not available [115].

In order to stimulate the adoption of biotechnological innovations also by small- and medium-sized companies and public research institutes, and their use in crops of minor economic importance worldwide, many researchers believe that it would be more appropriate to adopt a procedure assessment based on the product and not on the process, with evaluations conducted case by case, excluding the products of the new technologies described in this article from the regulation of transgenic plants so far produced [22, 104, 106, 110, 116-120]. That discussion is having conflicting outcomes in different countries, reflecting the general approach adopted in the regulatory procedures: based on the assessment of the technology, as is the case in Europe with the Directive 2001/18/EC, or the product, as it happens in North America [121]. Anyway, the potential benefits and disadvantages or risks of a larger use of biotechnologies for agriculture, health and the environment can be nowadays assessed more efficiently than in the past, sequencing the genome of modified plants and thoroughly profiling their metabolome [122]. In addition, the potential effects of the genetic modifications induced in crops can be analysed with predictive models based on '(Crop) Systems Biology' approaches [123-125].

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Publication History
Received 10th Mar 2017
Revised 27th Mar 2017
Accepted 06th Apr 2017
Online 30th Apr 2017