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The State of Art of New Transgenic Techniques in Plant Breeding: A Review

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Review Article

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ABSTRACT

The state of art of new plant breeding techniques has been developed at the back drop of progress accomplished in plant biotechnology to meet global challenges in food production. Some of the new plant breeding techniques are adopted by commercial breeders. The widely employed new breeding techniques are Cisgenics, Agroinfiltration and Oligonucleotide directed DNA Methylation. The crops developed by adopting these techniques are in commercial developmental phases. Few of the new techniques still at research level and could be close to commercialization are Reverse Breeding, Zinc Finger nuclease, Grafting on GM root stock and RNA dependent DNA methylation. The technology of Cisgenics includes transformation of gene belonging to its own genome. The emerging technologies to introduce controlled insertion of genetic material to targeted gene sequencing are Oligonucleotide directed methylation and Zinc Finger nuclease. The great technical potential and economical benefits, technical constraints, safety issues and also regulations of these new plant breeding techniques have been identified and are critically reviewed.

Keywords: Cisgenics; zinc finger; transgenesis; agroinfiltration; mirin; intragenesis; heterosis; achiasmatic.

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1. INTRODUCTION

Plant breeders are making a persistent effort to develop and adapt new novel technologies to speed up the plant breeding process considerably. Innovation in plant breeding is indispensable to meet global challenges − such as population growth and climate change. Indeed overall food production needs to be increased about 50% and doubled before 2050 to match the globally swelling human population [1]. Although traditional plant breeding accomplished many challenges in combating stress tolerance, pest and disease tolerance to enhance overall yield, it needs to address further improvements in its existing strategies.

The innovation in cell and tissue culture, utility of molecular marker technology so called smart breeding and genome mapping started in late 1990s undoubtedly facilitated the breeding process as parts of plant breeder's tool box.

During the last decades, plant breeder's tool box has been further explored and new plant breeding techniques are in the pipeline. The Institute of Prospective Technology Studies (IPTS) of the Joint Research Centre (JRC) of the European Commission critically analyzed the new plant breeding techniques such as zinc finger nuclease, reverse breeding, cisgenics, grafting on GM root stock, RNA dependent DNA methylation, and oligonucleotide directed mutagenesis which constitutes genetic modifications and if so whether above techniques come under the purview of European Commission GM regulations? [2]. There have been quantum increases in the scientific publications in the domain of new plant breeding techniques. One of the main limitations in current plant breeding is the introduction of right characteristics to the target plants. However, the above new plant breeding techniques offer new possibilities [3]. The following are the new plant breeding techniques which are reviewed comprehensively.

2. ZINC FINGER NUCLEASE (ZFN) TECHNOLOGY

Zinc finger nuclease technology has become a popular tool for efficient and site specific gene modifications in multiple cell types and organs [4]. Zinc finger nuclease exhibit promise in accelerating the efficiency of gene targeting by inflicting double stranded breaks in target genes which then stimulate the cells repair mechanism, and insertion of DNA [5-6]. Genome modification at the right place than at undesirable places is a challenging task. The ZFN nucleotide changes can be made on the targeted gene site or replacement of the existing gene region. Development in the ZFN technology can overcome conventional plant breeding problems of targeted genome modification and is a significant breakthrough in the field of DNA cloning [7-8] and plant biotechnology.

2.1 Zinc Finger how it Works?

Zinc finger nuclease simply called as zinc finger is a DNA binding protein that recognize specific sequences of three or four base pairs in the genome [5,9]. This 30 amino acid protein is stabilized by a zinc ion. The Cys2–His2 zinc-finger domain is among the most common types of DNA-binding motifs found in eukaryotes [10]. Although the zinc finger protein can recognize a sequence in genome, it fails to alter gene expression. Therefore, it is indispensable to couple it to an effector protein like nuclease, transcription factors and even to transcription repressors. The coupling of the endonuclease to a zinc finger then forms zinc finger nuclease (ZFN) which can recognize specific sequences and cut DNA at specific sites, the DNA breakage is then repaired by homologous recombination and non- homologous end joining [11]. Investigations show that there is an improvement of homologous recombination by as much as 50,000 times thereby making this technique popular in several fields [12-13]. Zinc finger coupled to a transcription factor can inhibit or stimulate specific gene expression and can be employed to regulate novel gene action in plants [14]. The versatility of this technique is facilitated by programmability of the DNA binding domains that are derived from zinc finger and transcription activator like effectors (TALE) proteins [10]. There are novel ways of introducing zinc finger into cells is in the form of a recombinant protein or the gene code for zinc finger effector protein with the help of vectors [15-19]. Some variants of Zinc finger nuclease (ZFN) technology have been reoriented in plant breeding such as ZFN—1 (delivery of ZFN genes into plant cells without a repair template) ZFN—2(Genes encoding ZFN, delivery into cells along with a repair mechanism [13-20].

Plant breeding objective is to develop new varieties with desirable traits by genetic modification using the conventional breeding approach. However, it may not be possible to

alter trait at several times due to lack of information on positional effect in the genome as it contains high and low transcriptional activation of genes. In addition to the above constraints of conventional breeding, agriculture biotechnology is also limited by the inefficiency of conventional random mutagenesis and transgenesis. Therefore, for plant breeders this emerging zinc finger technology enables the determination of insertion sites and facilitates greater degrees of gene expression. Recently, biotechnology company Dow Agro Science, adapted zinc finger in collaboration with Sangamo Biosciences [21]. Also Sangamo claimed that zinc finger technology can be used in rapid development of improved food crops [20]. The utility of zinc finger technology to achieve efficient insertion of genes for herbicide tolerance has been successfully accomplished in targeted genomic region of maize [20]. Site directed mutagenesis using ZFN has been performed in *Arabidopsis thaliana* as model system. Accomplishment of targeted mutagenesis of a transgene and nine endogenous soybean genes using ZNF based mutagenesis provided an efficient method for creating mutations in genes [22]. Similarly, rapid alteration of a soybean gene has also been modified by a refined zinc finger technique known as Context – Dependent Assembly ZFN (CoDAZFN). The efficacy of CoDAZFN will enable genome- wide alternations [23]. The possibility of delivering ZFNs directly as proteins were investigated [24]. However, direct delivery of ZFN proteins into cells was successfully accomplished in animal system [25].

3. REVERSE BREEDING (RB)

Reverse breeding is a novel technique in which there is a reversal of the method employed to produce a hybrid plant variety. Cisgenesis is genetic modification to transfer beneficial alleles from crossable species into a recipient plant. The donor genes transferred by cisgenesis are the same as those used in traditional breeding. It can avoid linkage drag; enhance the use of existing gene alleles. This approach combines traditional breeding techniques with modern biotechnology and dramatically speeds up the breeding process [1].

One of the significant insights in plant breeding have been linked to the performance of hybrid (F1) progenies which are typically superior in size, and growth characteristics in comparison to their homozygous parent, a phenomenon known as heterosis [26]. Breeders can assess heterosis by choosing inbreeding lines for crossing [27]. However, these hit or miss approaches may not be convenient to optimize the effect of heterosis [28]. Therefore, one of the alternative strategies is reverse breeding to fix complex uncharacterized heterozygous genome by constructing its complementary homozygous lines [29-30]. Reverse breeding is therefore designed to produce parental lines for any heterozygous parental lines through engineered meiosis [29,30]. The strategy is based on the reduced recombination in the selected heterozygotes by eliminating meiotic crossing over in developing spores and the production of double haploid (DH) offspring from such spores *in vitro* [31]. Finally, appropriate DH lines can be crossed to produce homozygous parental lines.

3.1 Reverse Breeding Method

Reverse breeding (RB) facilitates the production of homozygous plants using a heterozygous hybrid and can be reconstituted genetic composition of an elite heterozygous plant without using back crossing and selection [28,31,32,33,34]. The success of reverse breeding relies on achiasmatic meiosis or non −cross −over meiosis [30]. In most plants one or two cross overs takes place on chromosomal pairs. During crossing of homologous chromosomes, assembled and physically joined chromosomes form bivalents. Achiasmatic chromosomes remain as univalents. The elimination or reduction of crossover has been reported in several mutants [35-37]. The effective suppression of the meiotic process can be accomplished by inhibiting the expression of genes responsible for crossing over without altering the structure of chromosomes. Hybrid creation through reverse breeding was demonstrated in *Arabidopsis thaliana* [28]. The researchers have understood the genes and proteins responsible for crossing over. Examples of proteins involved in cross over was *Arabidopsis* ASY, rice ASY Homologue PAIR R2 [38-39] and other mutants proteins were dmc., Sds [40-41]. Recently, some other meiotic suppression genes have been discovered such as MER, MER2, MRE3, REC 102 and RoC 104 [42]. Reverse breeding can be achieved through RNA interference (RNAi) or post transcriptional gene silencing (PTGS SiRNA) [43]. Another approach is a chemical method or cross− over suppressor in which a chemical ‘’Mirin’ a potent inhibitor of cross over can be employed as exogenous applications to omite meiosis [44]. Utility of this mirin chemical does not only speed

up the applications of RB but is also free from any transgenic approach in achieving reverse breeding. Further, double haploids are achieved and it can be used to create homozygous parental lines. In addition to *in vitro* culture a completely novel approach for haploid induction has been developed by the genetic engineering of the centromere region [45]. Several well known protocols have been established in this direction [46-49]. RB technology however, is limited to crops with haploid chromosomes of 12 or less.

4. CISGENICS

Cisgenics are transgenic plants produced by plant transformation technique but the introduced DNA belonging to its own or related genome, compatible for crossing [50]. Cisgenesis is very useful technique to solve the problem of public issues regarding foreign gene of unrelated species [51].

Cisgenics employs genetic modification process by which genes can be conventionally bred, unlike transgenics, where sources of genes are from unrelated organism and in the case of cisgenics gene source is truly from within or wild plant relatives. The source of cisgenes is perfectly compatible even in conventional breeding. Hence, conceptual diversification can be seen in new breeding technique [52-53]. Cisgenics technique is more or less sandwiched between conventional breeding and transgenics. Several researchers argue that the logistic view on genetic modification of plants should be applicable to cisgenics since it contains introduced DNA belonging to the same genome or wild relative varieties [54]. Cisgenic technique can boost crop improvement process. Cisgenic plants are more safer than those produced by conventional breeding plants due to lack of linkage drag [55].

Some of the classical examples of the use of cisgenics in plant breeding are the production of blight resistant potato plants caused by *Phytopthora infestans* and cisgenic apple for resistance to *Venturia inaqualis* using genes from wild varieties and transfer them to high yielding varieties [56-57] and thereby eliminating linkage drag during gene stacking from wild varieties. Around five plant crops have been subjected for field trials produced through cisgenics [58].

There is an argument that cisgenics, used for traditional plant breeding should be exempted from regulation on genetically modified organism in a step by step approach. Much debate has been raised after recent calls for complete revised regulation of transgenic plants, which have only plant DNA inserted in to genomes of plant [59].

5. GRAFTING ON GM ROOT STOCKS

Grafting on GM root stock employs intended and unintended changes in the genome. In the context of its applications to plant breeding, grafting of a non – GM scion (ground vegetative component of plant) on to GM root stock is one of the key approach to produce chimeric plant. Some commercial applications have been witnessed from the use of GM root stock in grafting including improved root performance like root growth, disease resistant and other agronomic performance (Table 1). If a GM scion is grafted on to a non – GM root stock. Above ground parts such as seeds, edible region will be transgenic. When a non GM scion is grafted on to GM root stock, leaves, stem, flower and fruit would not carry the genetic modification with respect to changes in genomic DNA sequences [60–61]. The movement of DNA between root stock and scion has been evidenced. It was witnessed that the transfer of plastid DNA in a graft from root stock to the cells of the scion and vice versa [62]. With regards to unintended effects there have been movements of macromolecules such as recombinant proteins, hormones and siRNA transported from GM root stock to the scion. It is possible to silence gene expression in root stock by employing RNA interference. In grafted plants, the siRNA can mobilize through graft enable silencing signal which can effect gene expression in the scion [63-64].

6. RNA DEPENDENT DNA METHYLATION (RdDM)

RdDM facilitated breeders to produce plants, devoid of foreign DNA sequences or transgenes. As a consequence, there is no virtual genomic change but continued to modify the gene expression due to epigenetic. Plants use 24 nucleotide small interfering RNA (24-nt si RNAs) and long non coding RNAs (lncRNAs) to direct de nova DNA methylation and transcriptional gene silencing. The two proteins, DTF1/SHH 1 responsible for this process have been characterized [65]. Performing of grafting experiments in sweet cherry revealed that

siRNA travelled from GM root stock to non GM during assessment of siRNA mediated gene silencing of ring spot virus. The rational for use of RdDM in plant breeding is the silencing of specific genes in plants without any mutation or changes in nucleotide sequences. The actual mechanism involved is the methylation of promoter sequences of specific gene to alter their expression. Promoter methylation is carried out by the inverted repeat of genes encoding RNAs which are homologous to promoter regions are delivered in to plants [66-68]. It was observed that methylation patterns are meiotically stable in plant methylated promoter, resulting in desired trait and stable inheritance. The suggested population retains the desired trait in breeding programmes devoid of inserted gene [69].

7. OLIGONUCLEOTIDE DIRECTED MUTAGENESIS (ODM)

ODM is based on modification of targeted genes by introduction of mutation, reversal of an existing mutation or even induction of short deletion, by replacement of one or few base pairs in plant genome [70-71]. The ODM technology is also known as targeted gene correction, targeted gene repair and RNA mediated DNA modification. The oligonucleotide is usually made up of 20 to 100 nucleotides designed to pair with homology of targeted gene sequence in the genome.ODM can be delivered in to plant cells by particle gene gun method or electroporation in case of protoplast [72]. The technique has been focused towards generating base changes that result in selectable phenotype.

Table 1. New plant breeding applications based on published research papers

Technique	Crop	Trait	References
ZFN	Tobacco	Herbicide tolerance	Townsend et al. (2009) [80]
	Maize	Herbicide tolerance	Shukla et al. (2009) [20]
	Tobacco	Cleavage of model gene	Zeevi et al. (2008) [7]
ODM	Maize	Herbicide tolerance	Zhu et al. (2000) [71]
	Rice	Herbicide tolerance	Okuzaki & Toriyama (2004) [81]
	Tobacco	Herbicide tolerance	
	Oil seed rape	Herbicide tolerance	Kochevenkoa and Willmitzer (2003) [82]
			Ruiter et al. (2003) [83]
Cisgenics	Potato	Fungal resistance; black spot,	Park et al. (2009a) [84]
	Melon		Rommens et al. (2004) [54]
	Potato	Bruisse tolerance	Kuhl et al. (2007) [85]
		Fungal resistance	
RdDM	Maize	Male sterility	Cigan et al. (2005) [86]
	Potato	Modified starch content	Heilersig et al. (2006) [87]
Grafting on GM root stocks	Grapevine	Resistance against bacteria, fungi and virus	Aguero et al. (2005) [88]
	Water melon	Virus resistance	Han et al. (2009) [27]
	Orange	Fungal resistance; osmotic control	Mitani et al. (2006) [89]
	Cucumber	Virus resistance	Gal-on et al. (2005) [60]
	Tomato	Insect resistance	Mcgurl et al. (1994) [90]
	Walnut	Rooting ability	Vahdati et al. (2002) [91]
	Pea	Virus resistance	
Agroinfiltration	Tobacco	Production of vaccines in plants (hepatitis B, antibodies (HIV, hepatitis, Aprotin, vaccine production, HIV	Lombardi et al. (2009) [92]
	Tobacco	Screen for virus resistance	Lee et al. (2001) [93]
	Rice and Bean		Pogue et al. (2010) [94]
	Tobacco	Vaccine production	Steel et al. (2010) [95]
		Vaccine	

There have been persistent arguments that ODM should be exempted from genetically modified organisms (GMO) regulations since many plant breeding programs use mutagenic agents to improve varieties. However, in this case gene targeting for crop improvement does not involve insertion of any foreign DNA and therefore, should be placed under non GM category [72]. The ODM has proven to work on a variety of crop plants such as maize, wheat canola and banana when compared to other techniques like zinc finger, reverse breeding which are still working on model plants.

8. AGRO- INFILTRATION

In this approach one of the intended goals is the temporary expression of specific coding sequence without integration of introduced target DNA in the plant genome [73]. Agroinfiltration involves large scale introduction of foreign DNA copies directly on to plant tissues, mainly leaves using a liquid suspension of *Agrobacterium* sp containing genetic construct. As a result, gene expression and its product protein production exceedingly well in transgenic plants as a consequence of stable integration [74]. In plant breeding, this strategy can facilitate the rapid investigation of gene identification, its product functionality and more importantly, selection of plant genotype with the desired biological response to the introduced target gene or gene product in the context of favorable pathogen response. These agroinfiltered plants have been developed for the production of commercially important recombinant proteins [75]. Commercialization and near commercial-lization of several crops using above new plant breeding is tabulated in the Table 1.

8.1 Safety Issues of New Plant Breeding Techniques

The regulation of genetically modified organisms (GMOs) dates back to the year 1990 and revised regulations was again introduced in 2003 to regulate food crops. The new plant breeding technique cannot be distinguished with their conventionally produced breeding plants and therefore, considerable pressure to exempt these new plant breeding techniques from the clutches of GMOs regulation [76]. The changes in genome induced by ZFN1 can be comparable to the induced changes occurrence by mutation breeding. The only difference could be seen here is ZFN1 and ZFN2 technique induced changes is site specific. However, ZFN induced multiple site – specific changes cannot be ruled out [3]. Similarly, ODM does not result in other changes in the genome compared with mutation that occur as a result of mutagenic process. There have been substantial arguments to defend cisgenesis as it is safer than conventional breeding. It precludes the introduction of unwanted genes via linkage drag, otherwise it may lead to undesirable traits. In 2012, the European Food Safety Authority (EPSA) issued a report with their risk assessment of cisgenics and intragenics plants. They compared the hazards associated with plants produced by cisgenics and intragenesis with those obtained either by conventional plant breeding technique or transgenics. They concluded that the existing European guidelines for assessment of food and feed from genetically modified plants and guidelines on the environmental risk assessment of genetically modified plants were applicable for the evaluation of food and feed products derived from cisgenics and intragenics plants and did not need to be developed further [77]. The safety issues of RdDM is more flexible as it does not cause changes in the genome other than DNA methylation. Occurrence of methylation in nature induced by environmental conditions and also traditional breeding clearly indicated that this RdDM is more like a natural process. The safety issues relates to GM root stocks is that there might be unintended changes in gene, protein trait expression in the scion resulting from unwanted movement of protein, RNA from GM root to non GM scions. In case of reverse breeding, silencing of target homologous sequence by suppression of meiosis completely or not can be also accomplished by chemically, physical and environmental factor. In most of the techniques the transfer of T DNA fragment of *Agrobacterium* Ti plasmid is a serious safety issues. The two reports on cisgenics safety was published by COGEM [76] and Wageningen UR [78] proclaimed that integrated T-DNA in plants during cisgenic process is of foreign elements. However, reports of COGEM suggested that it is unlikely that T-DNA can cause any environmental risk. Moreover, the natural occurrence of T-DNA has been reported in plants [79].

9. CONCLUSION

The latest wave of innovation in new plant breeding techniques in the context of crop productivity has made a significant contribution to meet global challenges. The seven new techniques discussed above has great technical

potential, although need to be addressed in the process of complete commercialization. The proof of concept of the new plant breeding techniques has been accomplished by producing herbicide tolerance and insect resistance plants. While some techniques like grafting on GM rootstock have already been tested on many crop plants, others like ZFN technology have been tested mainly on model plants. It shows that all of the seven new plant breeding techniques have been adopted by commercial breeders. ODM, cisgenesis/intragenesis and agroinfiltration are the most used techniques and the crops developed with these techniques have reached commercial development phase I-III. The ODM has been proven technology as gene targeting system in many crop plants. The technique like ZFN technique, RdDM, grafting on GM rootstocks and reverse breeding are still at applied research level. It is estimated that many crops are close to commercialisation as several of the above mentioned techniques are more or less likely to be categorised under non GM.

It is difficult to bring out any essential difference between the products from the certain new plant breeding techniques such as ZFN 1, ODM, RdDM, grafting on GM stock, reverse breeding and agroinfiltration and from products obtained with conventional breeding and mutation methods. However, it is possible to identify products from the other new plant breeding techniques like ZFN 3, cisgenics and agroinfiltration provided some prior information is available. Although there is complete potential to commercialize all the technique, the regulatory uncertainty and the potentially high costs for risk assessment and registration are the main constraints at present.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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