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Review on New Plant Breeding Techniques

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Abstract: ***New plant breeding techniques are developed rapidly during the last decade due to advancements in genomic research, and play a crucial role in crop improvement. It is possible to accomplish targeted genome modifications in plants precisely, which was not possible though conventional breeding methods. These are potentially significant for the economical benefits in agriculture and food security. Techniques such as Cisgenics, Agroinfiltration and Oligonucleotide directed DNA Methylation are widely implemented and are in commercial development phase while techniques like Reverse Breeding, Zinc Finger nuclease, Grafting on GM root stock and RNA dependent DNA methylation are still at research level. Recent studies show the significant possibilities for improved crop varieties that are developed by various genome-editing techniques are difficult to obtain through traditional breeding methods. As compared to traditional breeding methods like chemical- or radiation-induced mutagenesis, used for crop improvement, the new breeding techniques are more precise and do not create multiple, unknown, unintentional mutations throughout the genome. The ambiguity in the regulatory frameworks of many countries is a major concern to be reviewed and addressed.***

Keywords: New plant breeding techniques, Site-directed nucleases, Cisgenesis, Grafting, Biosafety research

1. Introduction

Innovation in plant breeding is crucial to meet the challenges of global changes such as population growth and climate change. Because of the rise in world population and the need to protect the environment, the limited resources of land and water have to be used more efficiently for crop production [54]. Additionally, consumers demand healthy food and high value ingredients [64]. Therefore, plants with useful traits for pest resistance, disease, herbicide and stress tolerance and improved product quality characteristics have to be developed [19, 44].

In addition to a more efficient land, energy and water use, soil loss and greenhouse gas emissions per unit of agricultural output have been reduced during recent years by the use of improved varieties and agricultural techniques [52]. Further efforts are however needed and therefore plant breeders are making a persistent effort to develop and adapt new plant breeding techniques as an additional tool to meet these objectives and speed up the plant breeding process [48].

The discovery of the law of genetics by Gregor Mendel about 150 years ago enhanced the speed of plant breeding considerably [36]. The invention of cross breeding was followed by hybrid breeding in the 1930s, tissue and cell culture methods in the 1960s and recombinant Deoxy Ribonucleic Acid (DNA) techniques and genetic engineering in the 1980s [4]. So-called “smart breeding” started in the late 1990s with the use of molecular markers, genome mapping and sequencing [46].

The development of new techniques in plant breeding did not lead to the replacement of the older methods [25]. The use of all available technologies is essential for plant breeding. Conventional breeding techniques, transgenesis and new plant breeding techniques are essential components of what we could call the plant breeders’ toolbox [37].

Conventional Breeding

Plant breeding has been a trial and error exercise for many years, whereby new varieties are produced from a cross between parent plants or through self-pollination [18]. The process is based on identifying a desired characteristic in one plant – for instance higher resistance to a specific disease and crossing it with another plant which allows the desired trait to appear in the offspring [30]. However, a series of unwanted characteristics are transferred as well, which require several more breeding cycles in order to be replaced by desired traits [5]. This form of breeding takes many years. In order to speed up the process and allow for more precision and efficiency, new methods are needed [26].

New Plant-Breeding Techniques (Nbts)

Methods allow the development of new plant varieties with desired traits, by modifying the DNA of the seeds and plant cells [12]. They are called ‘new’ because these techniques have only been developed in the last decade and have evolved rapidly in recent years [39]. Based on assessments of the European Commission, the following plant-breeding techniques can currently be considered as the main NBTs [21]:

1) Site-Directed Nucleases (SDN) (including ZFN-1/2/3 and CRISPR systems);
2) Oligonucleotide Directed Mutagenesis (ODM);
3) Cisgenesis;
4) RNA-dependent DNA methylation (RdDM);
5) Grafting (non-GM scion on GM rootstock);
6) Reverse breeding;
7) Agro-infiltration.

These New Plant-Breeding Techniques, which have emerged as the result of advances in scientific research, enable more precise and faster changes in the plant’s genome than conventional plant breeding techniques, which use chemical and radiation processes to alter the genetic characteristics of plants [27].

As such, they have a significant potential for the plant

breeding and agri-food industry, as they entail technical advances, economic savings and the improvement of crop characteristics [23].

Technical advantages- some techniques (such as ODM and ZFN) allow site-specific and targeted changes in the genetic material of the plants, and for many of the techniques, the genetically modified code for the desired trait is only present in the first plant, but not in their offspring [2].

Economic advantages- the use of these techniques reduce the necessary time for plant breeding compared to conventional approaches (for which breeding can take up to 10 years), thus leading to lower production costs.

Crop improvements- include the resistance of plants to diseases and drought tolerance, which can lead to higher yields, as well as higher nutritional qualities and storage or processing qualities.

Advantages of NBTs

- Allow breeders to develop desired plant characteristics at a far more rapid pace
- Help develop resistance in plants to pests, thus reducing the need for pesticides
- Improve the precision and efficiency of the plant breeding process
- Provide more methods to increase food production in a sustainable manner
- Strengthen plants tolerance of disease and drought
- More efficient production, more food, and better use of water and other resources
- Benefits for farmers, consumers and the environment

1) Site-Directed Nucleases (SDN)

SDN refers to the general technology of using a DNA-cutting enzyme (nuclease) to generate a targeted break in the DNA [49]. The aim is to take advantage of the DNA break. The plant's natural DNA repair mechanism recognizes this break and repairs the break using enzymes naturally present in the cell [69]. The goal of SDN technology is to take advantage of the targeted DNA break and the host's natural repair mechanisms to introduce specific small changes at the site of the DNA break [56]. The change can either be a small deletion, a substitution or the addition of a number of nucleotides. Such targeted edits result in a new and desired characteristic, such as enhanced nutrient uptake or decreased production of allergens.

Description

SDN technology allows for specific and targeted mutations, thus enabling new plant varieties to be developed significantly faster than with traditional methods as no further breeding has to be undertaken to eliminate unwanted characteristics [5, 6].

The various applications of SDN are usually called SDN-1, SDN-2 and SDN-3, depending on the specific DNA break and repair process. Examples of SDN techniques include Meganuclease (MN), Zinc Finger Nuclease (ZFN), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR). SDN applications are divided into three techniques: SDN-1, SDN-2 and SDN-3.

- SDN-1 produces a double-stranded break in the genome of a plant without the addition of foreign DNA [21, 39]. The spontaneous repair of this break can lead to a mutation or deletion, causing gene silencing, gene knock-out or a change in the activity of a gene.
- SDN-2 produces a double-stranded break, and while the break is repaired by the cell, a small nucleotide template is supplied that is complementary to the area of the break, which in turn, is used by the cell to repair the break [21, 39]. The template contains one or several small sequence changes in the genomic code, which the repair mechanism copies into the plant's genetic material resulting in a mutation of the target gene.
- SDN-3 also induces a double-stranded break in the DNA, but is accompanied by a template containing a gene or other sequence of genetic material [21, 39]. The cell's natural repair process then utilizes this template to repair the break; resulting in the introduction of the genetic material [5].

SDN-1 and SDN-2 do not use recombinant DNA, do not lead to the insertion of foreign DNA. As such, they do not produce new plant varieties that fall under the scope of the GMO legislation [50]. In the case of SDN-3, the newly developed plant should fall under GMO legislation only if foreign DNA exceeding 20 bp is inserted.

1.1 Zinc Finger Nucleases (ZFNs)

ZFNs are a class of engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks at in DNA at specific locations [41]. They consist of a "zinc finger" domain (recognizing exact DNA sequences in the genome of the plant) and a nuclease that breaks double- stranded DNA [21]. The rationale for the development of ZFN technology for plant breeding is the creation of a tool that allows the introduction of site-specific mutations in the plant genome or the site-specific mutagenesis of genes followed by the cell's natural DNA-repair process [49]. Three variants of the ZFN technology are recognized in plant breeding as follows:

ZFN-1, ZFN genes are delivered to plant cells without a repair template. The ZFN binds to a specific DNA sequence and generates a site-specific DSB [41]. Gene repair mechanisms of the plant cell intervene to repair the break and generate site-specific mutations, which consist of changes of single or few base pairs, short deletions or insertions.

ZFN-2, ZFN genes are delivered to plant cells along with a short repair template, consisting of a DNA sequence homologous to the targeted area with the exception of a point mutation [41]. The ZFN binds to a specific DNA sequence and generates a site-specific DSB. Gene repair mechanisms of the plant cell intervene to repair the break and generate site-specific point mutations by copying the repair template.

ZFN-3, ZFN genes are delivered to plant cells along with a large stretch of DNA (e.g., a gene of interest). The ZFN binds to a specific DNA sequence and generates a site-specific DSB [41]. The ends of the DNA stretch are homologous to the sites flanking the DSB; therefore, the DNA stretch is site-specifically inserted into the plant genome.

1.2 TALENs (Transcription Activator-like Effector Nucleases)

It was developed in 2009, offer an easier and more accurate method of gene editing. Its first reported success came in 2012 when researchers at Iowa State University used the technique to develop disease-resistant rice. TALENs are more specific for particular genomic locations and thus cause fewer unwanted off-target effects than ZFNs [57]. The technique has also been used to create hornless cattle (avoiding the painful dehorning practice used by many dairy farmers) and soybeans with higher quality oil [61].

Recently, transcription activator-like effector nucleases (TALENs) have rapidly emerged as an alternative platform to ZFNs for genome editing and introducing targeted double-strand breaks (DSBs). TALENs are dimeric enzymes with a structure related to ZFNs and comprise a non-specific nuclease domain fused to a customizable DNA-binding domain [21]. It is composed of highly conserved repeats derived from the naturally occurring transcription activator-like effectors (TALEs) encoded by Xanthomonas proteobacteria, and contain DNA-binding domain then consists of an array of up to 30 modules, which are specific for a particular nucleotide sequence of 30 nucleotides. TALENs have generated much interest and excitement because they can be very easily and rapidly designed by researchers using a simple 'protein-DNA code' that relates modular DNA-binding TALE repeat domains to individual bases in a target-binding site [39].

1.3 CRISPR-Cas9

Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)-Cas9 is the latest and most influential of the gene-editing techniques, developed from the bacterial nuclease Cas9 (CRISPR associated 9), to target and edit DNA at specific locations [45]. It offers researchers a relatively low-cost, easy and fast option to engineer changes [28]. CRISPR is similar to a "biological word processing" system or molecular scissors that allows scientists to trim away weaknesses or insert strengths already found in the species being developed [38]. Researchers cut out a section of DNA. Then, one of two things happens: The loose ends are essentially glued back together, eliminating the undesired trait or weakness. Or a "repair" with a desired trait is inserted into the void.

CRISPR/Cas nucleases are guided to a particular genomic DNA sequence by a specific guide RNAs attached to the nuclease enzyme [1]. A naturally occurring model for such guide RNAs is provided by the RNAs directing Cas9, e.g. a complex between CRISPR-RNA (crRNA) and transactivating crRNA (tracrRNA) [31]. However the enzyme also accepts specifically designed synthetic guide RNAs modeled on the Cas9 guide RNA [60]. These synthetic guide RNAs direct the nuclease activity to intended target sequences in the genome, which are complementary to the synthetic recognition sequence of the guide RNA [29]. By this way a large number of different target sequences and thus different genome sites can be targeted.

2) Oligonucleotide-Directed Mutagenesis (ODM)

The ODM technology is based on site-specific oligonucleotide of 20-100 nucleotides that is induced into the cell by methods suitable for different cells types [66]. The genetic changes that can be achieved using ODM include the introduction of a new mutation (replacement of one or a few base pairs), the reversal of an existing mutation or the induction of short deletions [9]. The sequence of the oligonucleotide is homologous to the DNA sequence in the plant, except for the one or few nucleotides [74]. Therefore, after binding of the homologous genomic sequence, a mismatch pairing is created that is fixed by the repair system of the plant cell, resulting in a desired mutation in the plant's genome (the oligonucleotide is degraded by the cell after a short period of time). ODM may be used for targeted genome editing, e.g. to induce herbicide resistance by point mutation.

3) Cisgenesis and Intragenesis

Cisgenesis/intragenesis is very similar to conventional breeding, but involve transfer of an intact gene or DNA fragment between same plant species or closely related crossable plant species [10]. With this technique, a specific trait, such as disease resistance, is transferred from a same or closely related crossable plant species to another - without altering the plant's overall genetic makeup [48]. In the case of cisgenesis, the transferred gene is unchanged, whereas, for intragenesis, parts of a gene (e.g. regulatory elements) may be transferred [33]. Cisgenesis may lead to a new organism that is indistinguishable from a conventional cross. Intragenesis always leads to an organism that is not obtainable by conventional crosses [53].

Cisgenesis fastens the natural breeding process up to four times in a controlled manner, as the desired trait is absolutely introduced and no further breeding must be undertaken to abolish unwanted characteristics in the new plant variety [42]. As with conventional breeding, the donor plant must be crossable with the recipient plant, and the genetic transfer could also occur naturally as a result of crossbreeding [58].

Cisgenesis is a potential tool for the further improvement of elite crop varieties, especially in crops that are vegetatively propagated to maintain their genetic composition, such as apple, grape, potato, cassava and banana [43]. As cisgenesis produces varieties that are comparable to those produced by conventional breeding techniques, the European Food Safety Authority (EFSA) is of the opinion that cisgenesis presents the same level of safety as conventional breeding [21].

4) RNA-Dependent DNA Methylation (RdDM)

RdDM method facilitates modified gene expression by transcriptional gene silencing or promoter methylation without changing the genomic sequence [22]. The methylation patterns are induced by double-stranded RNAs

that are processed by various host enzymes of the RdDM machinery, including polymerases IV and V, Argonaute proteins and cytosine methyl transferases [63]. The epigenetic changes may be inherited and stable for at least a few generations [70]. The actual mechanism involved is the methylation of promoter sequences of specific gene to alter their expression [40]. Promoter methylation is carried out by the inverted repeat of genes encoding RNAs which are homologous to promoter regions are delivered in to plants [73]. It was observed that methylation patterns are meiotically stable in plant methylated promoter, resulting in desired trait and stable inheritance [62]. The suggested population retains the desired trait in breeding programmes devoid of inserted gene [71].

In plants, methylation patterns are meiotically stable. The change in the methylation pattern of the promoter, and therefore the desired trait, will be inherited by the following generation [55]. The progeny will include plant lines which, due to segregation in the breeding population, do not contain the inserted genes but retain the desired trait [60]. The methylated status can continue for a number of generations following the elimination of the inserted genes [22]. The epigenetic effect is assumed to decrease through consequential generations and to eventually fade out, but this point needs further investigation [62].

5) Grafting

Grafting is a traditional breeding method which involves physical attachment of two plants with different phenotypes producing a chimeric organism with improved cultivation characteristics [59]. Different techniques such as Transgenesis, cisgenesis etc. would be used to transform the rootstock and/or scion [15]. If a GM scion is grafted onto a non-GM rootstock, the resulting stems, leaves, flowers, seeds and fruits will be transgenic [21]. When a non-GM scion is grafted onto a GM rootstock, the resulting leaves, stems, flowers, seeds and fruits would not carry the transgenic DNA [21].

Rootstock can be transformed using conventional plant transformation techniques viz., Agrobacterium-mediated transformation and biolistic approaches [7]. Characteristics of a rootstock including rooting capacity and resistance to soil-borne diseases can be improved using genetic modifications, resulting in a significant increase in the yield of fruit [51].

This method may be used for expression of interfering RNAs (or RdDM) in the rootstock; these are systemically transported and may lead to transient or inheritable silencing of genes in the scion. Thus, the resulting seeds, fruits or offspring from such a scion do not contain any DNA of transgenic origin, whereas adventitious shoots regenerating from callus or rootstock may carry such transgenic DNA [68].

6) Reverse Breeding

Reverse breeding is a technique in which the order of events leading to the production of a hybrid plant variety is reversed [8]. The resulting breeding products are in essence identical to the initial best hybrid crop, which is the starting point for the breeding process [3]. This approach combines traditional breeding techniques with modern biotechnology and significantly accelerates the breeding process [47].

The following steps are involved in Reverse Breeding:

1) An elite heterozygous line is selected for its phenotypic characteristics.
2) Meiotic recombination is suppressed (e.g. through RNA interference, RNAi).
3) Gamete cells that do not contain the transgene are regenerated into homozygous, double haploid plants.
4) Parental lines are selected which together will reconstitute the initial heterozygous phenotype – only non-transgenic plants are selected.
5) The desired heterozygous genotype is obtained via crossing of the selected parental lines, resulting in final heterozygous plants being non-transgenic.

Although the Reverse Breeding process does involves recombinant DNA technology, the selected homozygous parental lines and their offspring are non transgenic [24]. The plant varieties that are produced as a result of this application are similar to those that can be produced through conventional breeding techniques [67].

7) Agro-Infiltration

Agro-infiltration is a technique in which plant tissues, mostly leaves, are infiltrated with an *Agrobacterium tumefaciens* suspension containing the desired gene(s) to be expressed in the plant genome [65]. Therefore, the desired genes are locally and transiently expressed at high levels [34].

Agro-infiltration can be used to 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 [11]. For example, agro- infiltration with specific genes from pathogens can be used to evaluate plant resistance [13]. The resistant plants identified in the agro-infiltration test might then be used directly as parents for breeding [35]. The progenies obtained will not be transgenic as no genes are inserted into the genome of the germline cells of the agro-infiltrated plant [14, 20].

There are three types of agro-infiltration which can be categorized based on the plant tissues and the type of gene construct infiltrated [72]:

1) “Agro-infiltration sensu stricto”: Non-germline tissue (typically leaf tissue) is infiltrated with non-replicative constructs in order to obtain localized expression in the infiltrated area [32, 34].
2) “Agro-inoculation” or “agro-infection”: Non- germline tissue (typically leaf tissue) is infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant [32, 34].
3) “Floral dip”: Germline tissue (typically flowers) is immersed into a suspension of Agrobacterium carrying a DNA-construct in order to obtain transformation of some embryos that can be selected at the germination stage. The aim is to obtain stably transformed plants. Therefore, the resultant plants are GMOs that do not differ from GM

plants obtained by other transformation methods [32, 34].

2. Conclusion

New Plant Breeding Techniques (NPBTs) are latest and advanced technologies in context of improved crop productivity and global food security [16, 17]. The above mentioned seven NPBTs have great technical potential in the commercialization of GM crops. Main achievements of NPBTs are accomplished by introducing herbicide tolerant and insect resistant plants. Although 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. Using these techniques, potential adverse effects are even less likely than in conventional transgenic plants or plants resulting from conventional breeding. The combination of various new techniques will allow precise genetic modification, resulting in plants that harbor as little recombinant DNA as possible or none at all. However, the decision whether a modified plant is regarded as transgenic or not is based on the techniques used to produce it. The NPBTs are very diverse in their technical impact and can be used in various combinations to produce new plant varieties. If a combination has one technique classified as GM technology the entire process and the resulting plant may be considered as GMO.

ODM, cisgenesis/ intragenesis and agro-infiltration are the most used techniques and the crops developed with these techniques have reached commercial development stage. 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 commercialization as several of the above mentioned techniques are more or less likely to be categorized under non GM. Therefore we require more practical handling of the NPBTs, such that modified plants that do not contain recombinant DNA are exempt from regulation and those containing recombinant DNA (which is not a hazardous) are de-regulated in some way as unintended side-effects are expected to be lower than in first-generation transgenic plants.

Different countries have different regulatory frameworks for the approval and release of plants developed by genome engineering technologies. Many countries have not yet decided whether, or which new technologies will be regulated for gene transfer. There is a need to develop some uniformity among countries related to regulatory policies and to establish standard guidelines for genome editing technologies.

There are issues related to regulatory frameworks of some countries, some countries does not have regulatory framework for genetically engineered plants while some are successfully implementing regulatory guidelines. In USA, no separate regulatory policy is being implemented for genetically engineered plants. For instance bacterial blight-resistance rice, amylopectin-rich – so called waxy maize and browning mushrooms are developed by genome editing, and kept outside of USDA-APHIS regulatory guidelines. In Canada, plants developed from conventional breeding, mutagenesis, transgenesis or genome editing comes under similar regulatory approval process. Argentina used to regulate all GE crops under general seed law, the first country among the globe to establish regulatory policy for such new technologies. In European Union (EU) countries, there are no such guidelines in the present GE regulations for the plants developed from NPBTs, but the final variety does not contain foreign DNA. New Zealand decided not to regulate transgene-free organisms through ZFN and TALEN by considering such techniques as conventional chemical mutagenesis. Similarly, countries like Brazil and Australia are not clear about their policies related to genome edited crops. So it is necessary to develop an effective regulatory framework which can address these challenges such countries.

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