RECENT ADVANCES IN TRANSGENIC BREEDING

Abstract

Abiotic stress is occurring more frequently and has reduced crop productivity as a result of the recent rapid climate change and rising world population. To improve plant genetics, transgenic breeding presents a viable alternative to traditional breeding. Transgenic breeding approaches allow us with access to identify the, miRNAs, candidate genes and transcription factors (TFs) that are involved in specific plant processes. Production of transgenic plants is reported in many crops but commercialization is limited to few selected crops, such as cotton (*Gossipium hirsutum* L.), corn (*Zea mays* L.), soybean (*Glycine max* L.) and canola (*Brassica napus* L.). Some of the recent advances in transgenic breeding gives opportunity to create better transgenes with precise gene insertion and specific character expression. One such development is clean gene/marker-free technology, which changes plants using two different vectors, one containing a transgene and the other a selectable marker gene. CRISPR-Cas-9 based base editing also offers the introduction of point mutation without generating double stranded brakes. Evolution in grafting process by placing non genetic engineered part on genetic engineered root stock is also modern approach in this field.

Keywords: candidate genes, miRNAs, Transcription factors

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I. INTRODUCTION

Transgenic breeding refers to the genetic engineering process used to improve domesticated animals, beneficial microorganisms, and crop plants for human economic use. Transgenic breeding methods have made significant strides in the last 20 years in terms of manipulating the genes to induce desired traits in transgenic plants. By adding advantageous foreign genes or inhibiting the expression of endogenous genes in crop plants, genetic engineering and plant transformation have been instrumental in improving crops. In last 22 years, the global area of transgenic crops has increased significantly from 1.7 million hectare in 1996 to 191.7 million hectare in 2018. The majority of the transgenic area is covered by soybean which is up to 48.2%. Maize stands second with 32% of total transgenic area followed by cotton with 13.5% of area. (Fig 1).

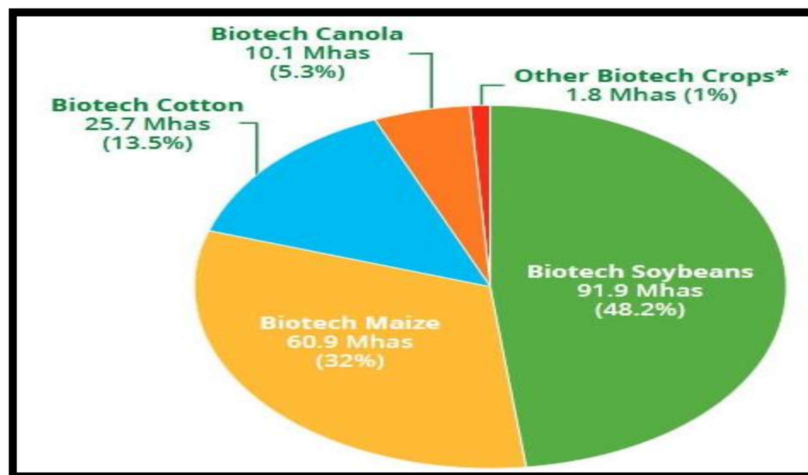

Figure 1: Transgenic crops in 2019 (area and adoption rate)

The basic four concepts on which transgenic breeding works are transgenesis, cisgenesis, intragenesis and genome editing. Transgenesis includes insertion of foreign gene from sexually incompatible species whereas, cisgenesis involves sexually compatible species. Genome editing refers to the introduction of targeted mutations at particular loci in the genome, whereas intragenesis refers to the insertion of recombinant genetic elements in which one or more components (gene, promoter and terminator) are isolated from different genes within the sexually compatible gene pool. (Figure. 2)

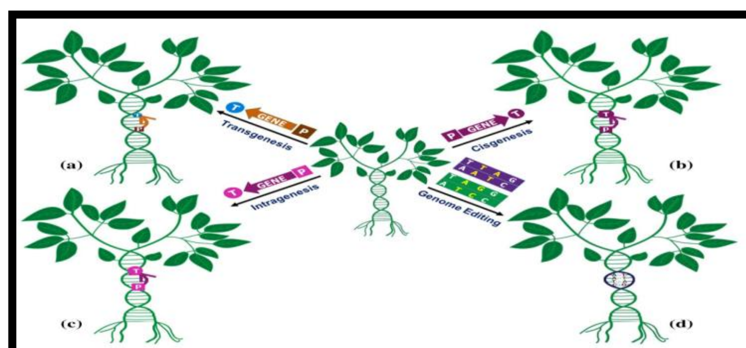

Figure 2: Illustration of various transgenic breeding techniques utilized for generation of improved crop plants.

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II. ADVANTAGES OF TRANSGENIC BREEDING

1. Rapid method of crop improvement
2. Overcome crossing barriers
3. Evolution of new genotypes
4. Genetic improvement of allogamous and autogamous crop plants
5. Effective for genetic improvement of monogenic characters

III. TRANSGENIC BREEDING APPROACHES

1. **Genetic transformation**
   - Physical method
   - Biological method
   - Chemical method
   - *In planta* transformation
   - Herbicide resistant gene now in the field

2. **Genome editing tools**
   - Site-directed nucleases (SDN)1/2/3
   - Zinc Finger Nucleases
   - TALE Nuclease
   - CRISPR/CAS 9

IV. THE RECENT ADVANCES

1. **Chloroplast genetic engineering:** In the *Nicotiana benthamiana,* the control of *Helicoverpa armigera* was more stable and effective when the overexpressing of dsRNA targeting the acetylcholinesterase gene was integrated into the chloroplast genome than it was integrated into the nuclear genome. Higher chloroplast numbers in each cell and its genome polyploidy allow the insertion of several transgene copies in a single cell, resulting in uniform and strong protein accumulation levels.

   Plant cells may be less harmful when recombinant protein is stored in transgenic chloroplasts as opposed to the cytoplasm. Additionally, most crops' maternal inheritance of chloroplasts prevents the transgene from being spread to other plant species through pollen.

   Furthermore, as chloroplasts facilitate the creation of disulfide bonds, they represent good bio-factories for mammalian proteins that require this type of folding. Transgenic plants can also be produced without any antibiotic resistance marker gene. The typical vector for the chloroplast genome transformation should contain gene of interest, selectable marker gene driven by an organelle-specific promotor and 50- and 30-UTR (UnTranslated Region). (fig. 3)

   Chloroplast engineering is also helpful in various fields *viz.* Therapeutic protein, biofuel, phytoremediation, improved crop production and industrial enzymes.

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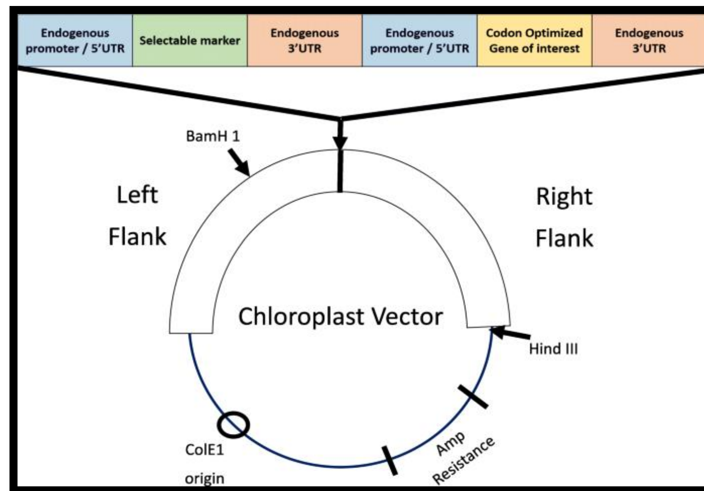

Figure 3: Typical Vectors for Chloroplast Genome Transformation

2. **Base editing:** The risk of using CRISPR or other techniques to introduce many double-strand breaks simultaneously inside the genome is that the edited cells' inherent DNA repair mechanisms will make mistakes, bringing modifications into the genome that could result in cellular transformation and cancer. Base editing is a novel technique that can produce gene knockouts or fix specific mistakes or mutations in the DNA of healthy cells. This many gene knockouts can be accomplished with CRISPR, ZFNs, or TALENs, but doing it by the introduction of double-strand breaks is not the best method. Base editing does not require the creation of double strand breaks in order to change a base pair, thus lowering the possibility of genome-wide changes like chromosomal translocations. This has a very attractive safety profile as the preferred gene editor for cell therapy. (Fig. 4)

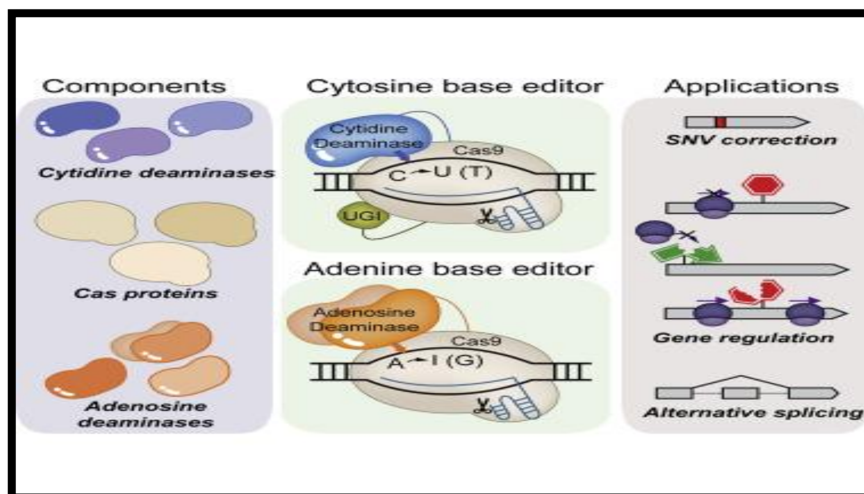

Figure 4: Base Editing Procedure

3. **Clean gene/ marker free technology**

   - **Need for marker free transgenic:** After selection events, marker genes typically have minimal agronomic relevance. The protein products of such genes could be toxic to human/animals. The environment could be harmed if these marker genes are

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transferred from transgenic crops to other organisms. One should employ the same marker in subsequent transformations when a situation calls for more transformations into cultivars and a transgenic plant possesses a specific marker gene. For public acceptance of transgenics, keeping in mind ecological and food safety, marker free transgenics should be developed. Clean gene technology is the process of creating transgenic plants by removing "problematic" selectable marker genes from the genome of the transgenic plants or by starting the transformation without using selectable marker genes using a marker-free vector.

This procedure can be carried out through four methods

- **Co-transformation and segregation:** Selectable marker gene and gene of interest are introduced on separate T-DNAs present in to two different agrobacterium strains (A) On separate vector in same agrobacterium strain or (B) on the same vector. The two genes may alternatively be transferred directly using a technique like particle bombardment. After sexual reproduction, plants with only the desired gene can be obtained if the selectable marker gene and the gene of interest are integrated at unlinked locations. (Fig. 5)

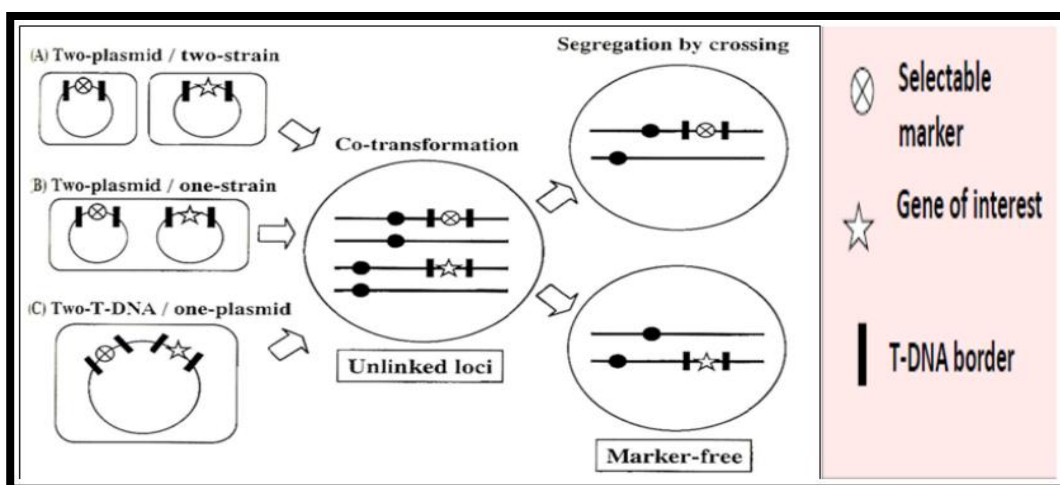

Figure 5: Co-transformation and Segregation

- **Site specific recombination:** Site-specific recombination techniques can be used to eliminate selected marker genes. The *cre* lox system is derived from the bacteriophage P1 and consists of two components 1. Two loxP sites, each having 34bp inverted repeats cloned in direct orientation flanking the DNA sequence 2. The *cre* gene encoding a recombinase protein that specifically binds loxP sites and excises the intervening sequence by catalysing a cross over between the repeated lox sites. The strategy involves the generation of plants that express the *cre* gene and crossing them with the plants in which the selectable marker gene is introduced between two lox sites. In the F_1 generation, the selectable marker gene is excised due to expression of *cre* recombinase. The *cre* gene is then segregated away in the subsequent generation. (Fig. 6) Besides *cre* lox system other site-specific recombination system include, the flippase recognition target or *frt* system.

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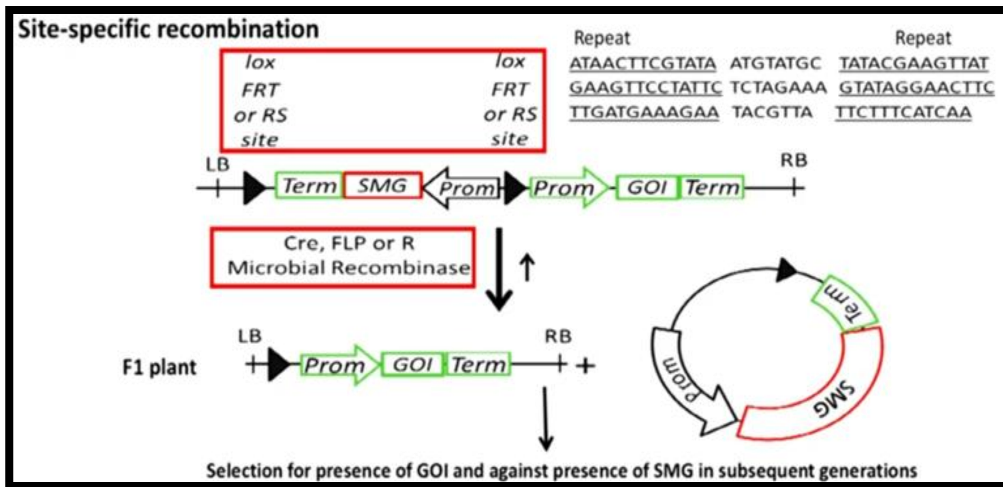

Figure 6: Site Specific Recombination

- **Transposon based marker excision:** This system is primarily based on the fact that the DNA sequences located in the AC-DS repeats are excised along with the AC-DS element. Agrobacterium mediated transformation is carried out to transfer the selectable marker gene that is cloned as a part of transposable element *i.e* AC and the gene of interest, with both genes laying within the T-DNA borders. Alternatively, the AC can be introduced by crossing another transgenic carrying AC. Re-insertion of a changed element and a selectable marker gene may occur as a result of transposition. A free insertion takes place in an unlinked location, and after crossing and segregation, marker-free progeny may be obtained. Alternately, the loss of the selectable marker gene may also come from the excision of the modified transposable element if no re-insertion takes place. (Fig. 7)

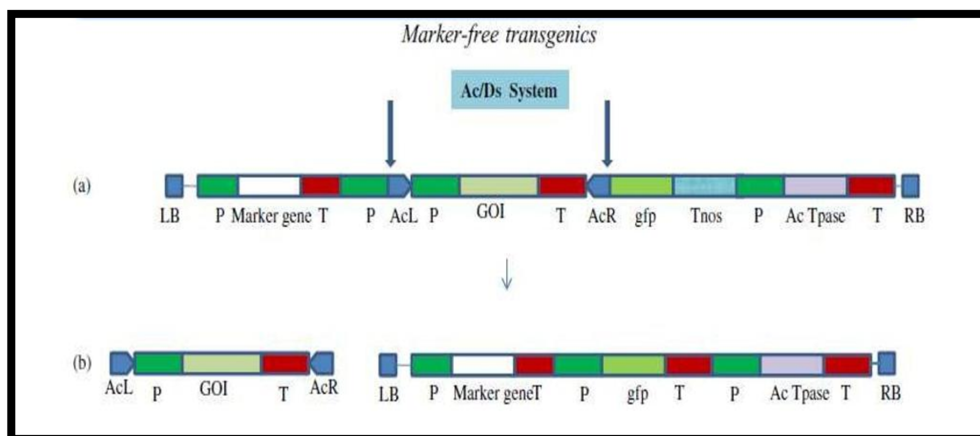

Figure 7: Transposon Based Marker Excision

- **Homologous recombination-based marker excision:** Homologous sequences may be the plastid promotors *prrn* and transcription terminators present on the vector used for the insertion of transgene. Recombination *via* the plastid promotors or the transcription terminators repeats yields two stable marker free transplastomic DNA carrying only the gene of interest 1 *via* recombination event R2 (Fig. 8) or the gene of

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interest 2 *via* recombination event R1. While the gene for the selectable marker aadA, coding for spectinomycin resistance was removed. The promotor or terminator sequences were not repeated in the final product.

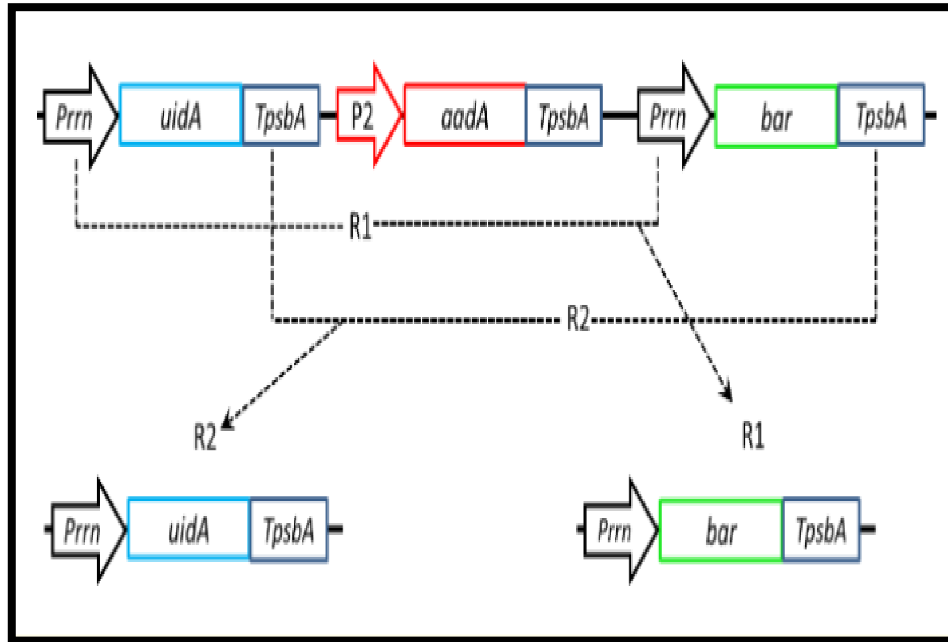

Figure 8: Homologous Recombination-Based Marker Excision

These processes can be hence summarized as: due to the undesirable effects a need to avoid use of selectable marker genes in transformation methods or to remove selectable marker genes after their use in selection of transformations is over such that the transformed plants finally do not express them. Selectable markers can be avoided totally and identification or transformations could be done using pcr but it is very tedious or markers that enable selection without being potentially hazardous could be used like mannose phosphate isomerase which enable growth on mannose medium. The selectable marker gene can be eliminated using techniques like co-transformation and segregation, site-specific recombination, or transposon-based excision if transformants are produced that carry both the gene of interest and the selectable marker gene.

- **Trans-grafting:** A non-transgenic scion is grafted onto a transgenic rootstock in a process known as trans-grafting. The vascular transmission of RNA, hormones, or signalling proteins confers some advantageous rootstock traits, such dwarfing or disease resistance, to the scion, but the shoot, leaves, and fruits are transgene-free. Non-transgenic grapevine scions were grafted onto rootstocks that were designed to produce antimicrobial peptides and proteins that prevent the breakdown of cell walls. (Fig. 9) Therefore, transgenic rootstocks can increase the yield of fruit trees with significant commercial value, yet the fruits and seeds do not contain any exogenous DNA.

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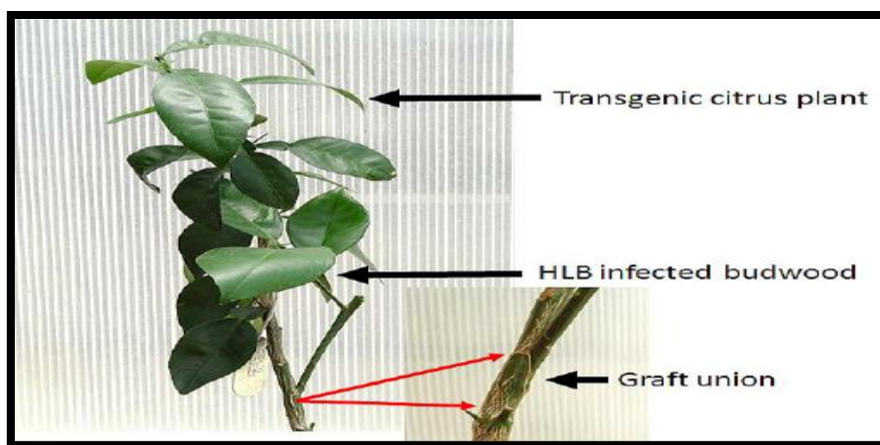

Figure 9: Transgenic plant challenge *via* grafting with HLB-infected sweet orange budwood after 8 months of inoculation.

V. FUTURE DIRECTIONS

1. **Zygotes and pollen as delivery targets:** Plant components like zygotes and pollen may prevent protoplast renewal. Gamete fusion, the joining of separate sperm and egg cells, can produce rice zygotes. Early gamete fusion results in immature cell walls, which enables PEG-mediated transfection of preassembled CRISPR/Cas9 RNPs. 14-64% of the plants produced from the zygotes had CRISPR-induced alterations after 30–40 days of cultivation. This strategy shows promise and might be used with other species that have protocols for gamete fusion and regeneration. A lot of the issues with tissue culture and regeneration could be avoided by manipulating pollen. Many plant species' pollen grains are theoretically suitable to the delivery of preassembled RNPs utilising nanotechnological methods since they can pass through apertures of 5–10 m in diameter.

2. **Nanoparticles for cargo delivery:** DNA, RNA and proteins have been successfully delivered into plant cells using nanoparticles (<100 nm). Several dicot plants, including cotton, pepper, pumpkin, and cocozelle had exogenous DNA plasmids introduced into their pollen grains using Polyethyleneimine PEI-coated Fe3O4 magnetic nanoparticles. In a procedure known as magnetofection, the DNA-loaded nanoparticles were mixed with pollen in solution and exposed to a magnetic field to facilitate the flow of the particles to the bottom of the receiver and into the pollen grains. GMO seeds were produced through artificial pollination utilising pollen that had been magneto-infected. Using this method, preassembled RNPs rather than DNA could be added to pollen.

VI. LIMITATIONS IN TRANSGENIC BREEDING

1. Low frequency
2. It requires high technical skill
3. Transgenic breeding is unable to transfer polygenic traits
4. It can have adverse effect on natural evolution.
5. Position effect
6. Pleiotropic effect
7. Instable performance

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