

## A Brief Overview for the Development of Herbicide-Resistant Sugarcane Transformation Approaches

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### ABSTRACT

Weeds are undesirable plants grown in a situation that can significantly decrease the yield of desirable plants such as sugarcane. Sugarcane (*Saccharum officinarum*) is the 2<sup>nd</sup> vital cash crop of Pakistan, placed at 6<sup>th</sup> position in world cane acreage and 15<sup>th</sup> in sugar production. Sugarcane occupies a significant share in the national economy and development of a country. Several factors contribute to the lower production of sugarcane in Pakistan, but the quality and quantity of sugarcane production are mostly damaged by weeds. The development of herbicide-resistant sugarcane that is highly resistant to several herbicides is produced by genetic transformation, site-directed mutagenesis, and plant breeding. Broad-spectrum herbicide-resistant plants are developed to resolve this issue by the insertion of CP4 EPSPS (Glyphosate tolerant gene; 5-enolpyruvulshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4) into sugarcane which provides them with the ability to survive after the spray of glyphosate. The most common herbicide-resistant technology is glyphosate-resistant technology which gives broad-spectrum weed control feasibility along with flexibility in the application time of herbicide. Numerous methods of genetic transformation are available for the insertion of foreign DNA into plant cells like *Agrobacterium*-mediated transformation, micro-projectile bombardment (gene gun), and protoplast transformation. Glyphosate tolerant gene expression is shown by three sugarcane cultivars (CPF-213, SPF-234, and HSF-240). Transgenic sugarcane plants expressed glyphosate-resistant genes are highly stable against herbicide to control weeds. Several farmers in many countries have rapidly and extensively utilized herbicide-tolerant crops due to low production and labor costs, huge profit, increased weed control, and many environmental benefits.

**Keywords:** Herbicide-Resistant, Sugarcane, Gene Gun, *Agrobacterium*-mediated Transformation, Weedicide, Genetic Transformation.

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## INTRODUCTION

Weeds are undesirable plants grown in a place that can significantly decrease the yield of desirable plants such as sugarcane (Badhai et al., 2021). Sugarcane (*Saccharum officinarum*) is the 2<sup>nd</sup> vital cash crop of Pakistan, placed at 6<sup>th</sup> position in world cane acreage and 15<sup>th</sup> in sugar production. Sugarcane occupies a significant share in the national economy and development of a country (Qamar et al., 2017; M. Raza et al., 2013). Sugarcane provides basic raw material to 84 sugar mills to produce sugar which is the 2<sup>nd</sup> major agro-industry of Pakistan (Raza et al., 2013). According to the Economic Survey of Pakistan 2019–2020, sugarcane contributes 2.9% value to agriculture and 0.6% of the total Gross Domestic Product of Pakistan is also due to sugarcane production (Economic Survey of Pakistan, 2019-2020).

The average sugarcane yield of Pakistan is only 46–48 t/ha which is much lower as compared to the US (80 t/ha), India (69 t/ha), and Egypt (107 t/ha) (Nagaraju et al., 2014). Herbicides' application is a well-recognized requirement for the most critical plant growth phase and crop regeneration in sugarcane (Ishida et al., 2021). However, the selection and application of herbicides must be carefully monitored because these herbicides can disturb the various essential biological processes such as photosynthesis, biosynthesis of amino acids which are common in both crops and weeds especially in sugarcane cases in which most of the weeds also belong to gramineous species (Dong et al., 2020). The development of herbicide-resistant sugarcane that is highly resistant to several herbicides is produced by genetic transformation, site-directed mutagenesis, and plant breeding (Han et al., 2014).

Several factors contribute to the lower production of sugarcane in Pakistan such as

mismanagement of cultural practices, climatic effects, deficiency of irrigation water, delay in sowing or harvesting, use of the uncertified or improper seed, nutrition imbalance, poor quality control in ratooning, attack of insects, pests or diseases but the most important is the large number of weeds that covered the most of the area of sugarcane cultivated fields which results in major loss of sugarcane yield in terms of quality and quantity (Nagaraju et al., 2014, Han et al., 2014).

Most weeds reduce the sugarcane yields because these weeds act like competitors to attain moisture, nutrients, and light for their growth and survival (Odero et al., 2014; Rizwan et al., 2015). One specific strategy which is usually used to control weeds is the use of unique herbicide against the specific type of weed but it is a highly expensive approach for the farmers. Broad-spectrum herbicide-resistant plants are produced to combat weeds by the insertion of CP4 EPSPS (Glyphosate tolerant gene; 5-enolpyruvulshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4) into the plant of interest like sugarcane.

Transgenic plants can survive after the spray of glyphosate, but weeds do not survive in this condition. The complete removal of weeds is the successful objective of herbicide-resistant transgenic plants. Broad-spectrum herbicide-tolerant crops are broadly and quickly accepted and cultivated by farmers in many countries because these crops have greater herbicide-resistant power, need few labors, least production costs, greater environmental advantages, and greater earning or profit.

The well-known broad-spectrum herbicide is Glyphosate (N-phosphonomethyl glycine) which is commonly used to eliminate unwanted and undesirable plants i.e., weeds (Qamar et al., 2015). The most common herbicide-resistant technology is glyphosate-resistant technology which

gives broad-spectrum weeds control feasibility along with flexibility in the application timing of herbicide. The glyphosate-resistant EPSPS enzyme contributes to high-level herbicide resistance in most transgenic plants (Qamar et al., 2015; Zameer et al., 2015).

In many plants including many weeds, glyphosate inhibits the chloroplast localized EPSP synthase (5-enolpyruvylshikimate- 3-phosphate synthase) that results in failure of aromatic amino acid synthesis but the overexpression of the EPSPS gene in transgenic cells gives them the glyphosate resistance (Achary et al., 2020). Numerous transformation approaches are available for the insertion of foreign DNA into plant genome like *Agrobacterium*-mediated transformation, micro-projectile bombardment (gene gun), and protoplast transformation but these methods have also some disadvantages.

The latest research has developed certain advanced transformation methods included infiltration, electroporation of cells and tissues, electroporation, microinjection, pollen-tube pathway, silicon carbide (SiC), and liposome-mediated transformation. Most alternative transformation methods have low transformation efficiency and hence, they have less or limited popularity except the infiltration and silicon carbide (SiC) mediated transformation (Aly, Lubna, & Bradford, 2021).

## METHODS OF TRANSFORMATION

There are various transformation methods currently available that can be divided into two major groups such as direct and indirect methods. The direct methods are those transformation methods that do not use bacteria as mediators or mode of transformation while indirect methods are usually established on the plasmid construct introduction into any specific target by utilizing bacteria such as *Agrobacterium tumefaciens*. In the indirect

transformation methods, numerous types of herbicide or antibiotic-specific resistance genes are principally introduced into the T-DNA portion of the specialized binary vectors and hence, these genes are used for the selection of transgenic plants. The most used selectable antibiotic-resistant marker genes are neomycin phosphotransferase (NPTII) and hygromycin phosphotransferase (HPT) genes having resistance against kanamycin and hygromycin respectively in plants but numerous other regularly used selection systems exploit several other herbicides such as bromoxynil, glyphosate, gluphosinate, and phosphinothricin (Hwang et al., 2017).

*Agrobacterium*-mediated transformation is the leading method, currently used in the applied research and biotechnology field (Din et al., 2021; Sufyan Tahir et al., 2021). However, the utmost applied direct methods in basic research are micro-projectile bombardment and protoplast transformation due to low transformation efficiency in monocots by *Agrobacterium*-mediated transformation (Zhang et al., 2013). Many direct transformation methods have developed up till now which include electroporation, liposome-mediated transformation, microinjection, infiltration, electrophoresis of embryos, silicon carbide mediated transformation, pollen tube mediated transformation. These methods are mostly developed as the most effective possible solutions against those species that were difficult to transform due to recalcitrant nature like few legumes and monocots because *Agrobacterium*-mediated transformation was mainly not available for such group of plants at that time (Javied et al., 2021).

### **Agrobacterium Mediated Genetic Transformation**

The genetic transformation of plants mainly relies on *Agrobacterium tumefaciens* which is the most powerful vector

for the targeted gene delivery into the plant of interest. In this method, the transferred DNA can integrate into the genome of the plant inside the plant nucleus which results in stable transformation having the potential to inherit in the next generations of the plant (Yaqoob et al., 2020). The other possibility is the transient transformation in which the transferred DNA remains in the plant nucleus transiently but does not integrate into the plant genome. However, the foreign DNA still has the potential to transcribe and translate to generate the desired gene product i.e., protein of interest. This unique transformation method has numerous advantages like easy to use, economical or relatively inexpensive, the transformation of low DNA copy number, and the specified DNA insertions into the plant chromosome (Oropeza-Aburto et al., 2020).

In several physical and chemical transformation methods, there are complex DNA integration arrangements that commonly result in epigenetic transgene silencing and genetic transgene instability in the transformed plants (R.M. Shelake et al., 2019). Hence, we can say that this *A. tumefaciens* mediated transformation is still the most common and widely utilized method for genetically modified crops and transgenic plants production due to these main restrictions and disadvantages of chemical and physical transformation methods (Din et al., 2021; Sufyan Tahir et al., 2021; Yaqoob et al., 2020).

### **Microprojectile Bombardment/Biolistic /Gene Gun**

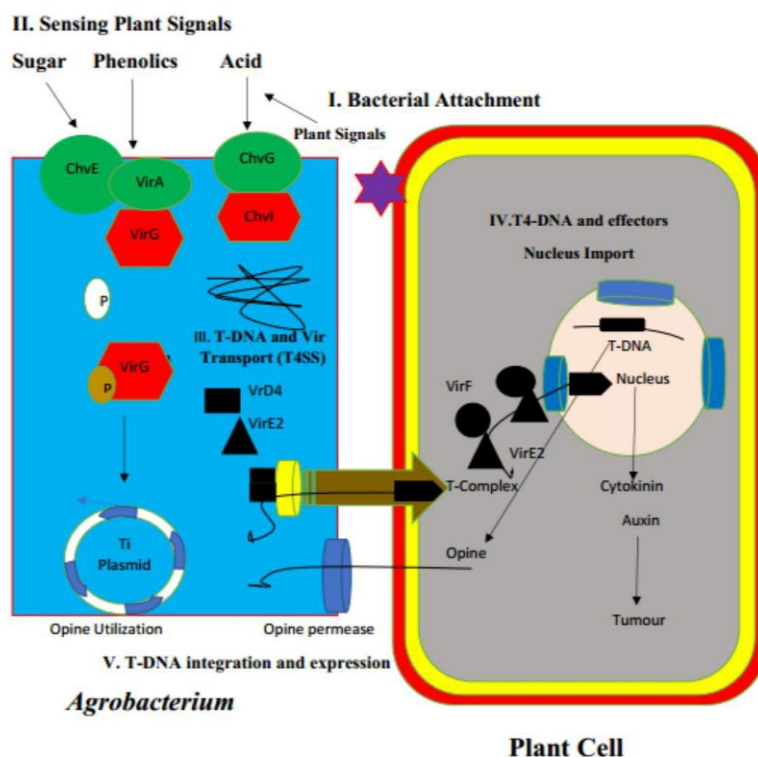
Biolistics, also called micro-projectile bombardment, is considered the best substitute for Ti plasmid (*Agrobacterium tumefaciens*- mediated) DNA delivery systems for plants (Gavit et al., 2020). In this method, DNA-coated spherical tungsten or gold particles are accelerated to a very high speed through a particle gun so

that these DNA-coated particles can penetrate plant tissues (Demirer et al., 2017). Therefore, this method can be utilized to introduce any foreign DNA into numerous plant tissues of many plant species having the advantage that no need to clone the gene of interest into a unique vector for transformation purposes. Unfortunately, this transformation process results in the insertions of many DNA copies in plant cells which can ultimately lead to the loss of DNA molecular integrity, and the size of DNA is also a big limitation of this transformation method (Vij et al., 2018).

### **Herbicide Resistant Sugarcane Transformation**

The crop yield can be reduced by the presence of unwanted plants during sugarcane plantations. The major losses in quality and quantity are due to the presence of weeds (Ali et al., 2014). During growth season weeds the utilization of a specific herbicide for a specific type of weed can minimize the weed problem, but the cost is too much for farmers. Broad-spectrum herbicide-resistant plants can reduce the cost problem as well as the complete removal of weeds. These plants can be obtained by the introduction of CP4 EPSPS (Glyphosate tolerant gene) into a crop plant that will survive after glyphosate spray. The farmers in leading countries have rapidly and extensively utilized herbicide-tolerant crops due to low production and labor costs, huge profit, increased weed control, and many environmental benefits. Glyphosate is a broad-spectrum herbicide that has been widely utilized on large scale to kill weeds. Glyphosate-resistant technology provides broad-based weed control with flexibility in application timing. Transgenic plants have a high level of herbicide resistance due to the presence of glyphosate resistant EPSPs enzymes which have been isolated from *Agrobacterium* spp. strain CP4 (Zameer et al., 2015). In plants, EPSP synthase (5-





**Figure 1.** Key steps of *Agrobacterium*-mediated transformation in the plant cell. (I) *A. tumefaciens* attaches to the plant cell. (II) *A. tumefaciens* senses plant signals and regulates virulence genes, and subsequently transduces the sensed signals. (III) T-DNA and virulence proteins generate and transport from the bacterial cell into the plant cell. (IV) T-DNA and effector proteins nuclear import into the plant cell. (V) Integration and expression of T-DNA in the plant genome.

enolpyruvylshikimate-3-phosphate synthase) is a chloroplast localized enzyme that can be inhibited by glyphosate leads to the failure of aromatic amino acid synthesis. The glyphosate tolerance in the transformed plants can be distributed by the overexpression of the EPSPS gene in cells (Fang et al., 2018). Gene gun is the best direct method for gene transformation and most widely and commonly used for gene transformation. In this method, the bombardment of more than one gene can be employed simultaneously in the host plant as well as an efficient, tissue-specific, and rapid method for gene transformation (Yaqoob et al., 2020). Genotypic variability was responsible for a higher degree of variance between callus formation and regeneration among different varieties. The successful transformation of genes in sugarcane plants was confirmed through

PCR by using gene-specific primers (Muzaffar et al., 2015). Insect-resistant and glyphosate-tolerant gene expression was shown by SPF-234 sugarcane cultivar among three (CPF-213, SPF-234, and HSF-240) cultivars. Transgenic sugarcane plants showed these expressions were most stable against herbicide to control weeds and insects (Qamar et al., 2017).

## CONCLUSION

Sugarcane (*Saccharum officinarum*) is produced on a big scale in tropical and subtropical regions of the world used as raw material for sugar and several industrial products. The sugarcane yield is reduced due to the presence of numerous undesirable plants i.e., weeds. The major losses in sugarcane quality and quantity are due to the presence of weeds. During the growth, season weeds compete with

sugarcane for light, moisture, and nutrients and ultimately reduce the sugarcane yield. The utilization of a specific herbicide for a specific type of weed can minimize the weed problem, but the cost is too much for farmers. Broad-spectrum herbicide-resistant plants can reduce the cost problem as well as help in the complete removal of weeds. These plants can be obtained by the introduction of CP4 EPSPS (Glyphosate tolerant gene) into a crop plant that will survive after glyphosate spray.

The transformation of an herbicide-resistant gene into the germplasm of sugarcane has been done through various genetic engineering tools. Numerous transformation approaches have been applied for the insertion of foreign DNA into plant cells: like *Agrobacterium*-mediated transformation, micro-projectile bombardment (gene gun), and protoplast transformation. Conventional plant breeding methodologies along with basic traditional biotechnological approaches are broadly used to improve the desired traits of selected varieties such as crop yield. Unfortunately, some vital crop traits such as resistance to herbicides, insects, or pests are usually not present in several sugarcane cultivars. Therefore, the transformation of herbicide-resistant genes into the sugarcane genome is the only suitable approach to overcome weeds and significant sugarcane yield.

Genome editing through CRISPR/CAS9 technology is the state-of-the-art technology for the mutated DNA sequences within the genome. CRISPR/CAS9 is a microbial defense system in which bacteria use Cas9 protein to cut the double-stranded DNA of invading viruses with the help of guide RNA. A powerful site-specific genome editing tool CRISPR-CAS9 has brought a revolution in the field of genome engineering in the past years. CRISPR/CAS9 system will be adopted by constituting CRISPR constructs to induce resistance against weedicides in sugarcane

crops. Moreover, a multiplex genome editing system could be achieved by expressing Cas9 along with multiple guide RNA for respective knockdown of the mechanisms affected by weedicides in sugarcane crops. The genome editing technologies, CRISPR/CAS9 technology is the only potential future to induce resistance against weedicides in different crops. Moreover, protein engineering technologies, site-directed mutagenesis, can be used to induce resistance in sugarcane plants. The development of herbicide resistance sugarcane plants will have a significant impact on the national economy and farmers.

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