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Development of Multigenic Sugarcane to Decrease the Production Cost in Pakistan

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Abstract

Due to higher amount of sucrose, sugarcane is grown commercially. In order to save sucrose yields, various studies have been designed to develop resistance in sugarcane against weeds and stem borers. In this study, two problems had been addressed by genetic manipulation of sugarcane to make them resistant against both herbicides and insects by expressing glyphosate resistant gene (CEMB-*GTGene*) and borer resistant genes (CEMB-*Cry*1Ac and CEMB-*Cry*2A) under control of NOs terminator and maize ubiquitin promoter. Mortality percentage of shoot borers *Chilo infuscatellus* was determined by assessing the *Cry* proteins through insect Bio-toxicity assays. Results showed that in 80 days old transgenic plants, 100% mortality rates of *Chilo infuscatellus* have been found showing that there was high resistance in transgenic sugarcanes against shoot borers and sufficient gene expression to fully resist target pests. Weed management was done by glyphosate spray assays. 70% to 76% of the transgenic plants were identified to be glyphosate resistant (3000 ml/Ha) in V_1 generation while 100% tolerant in V_2 generation. Thus, this transgenic sugarcane will help to boost sugarcane yield in the country as it now successfully provides resistance against both stemborers and glyphosate herbicides.

Introduction

Sugarcane is considered to be the world's significant cash crop as it is being cultivated around the globe in 58 countries [2] and 26.9 million hectares of area is used for sugarcane cultivation worldwide [1]. The 80% of world's sugar need is fulfilled by sugarcane *via* chemically synthesized sweetener known as sucrose [3]. A wide range of products are obtained from sugarcane like chemicals, biofuels, fibers, paper, beverages, detergents, insecticides, industrial enzymes, plastics, paints, pharmaceutical products, synthetics, chipboard and industrial chemicals like dextran, furfural and alcohol [4]. Sugarcane contributes to 0.7% GDP and 3.4% of agriculture sector and is cultivated on ~1.3 million hectare area in Pakistan [5]. 37% of the agriculture production in Pakistan is lost out of which 13% is because of insects [6]. Sugarcane crop is destroyed by ~1300 insect pests all over the world and by 61 insect species in Pakistan [7]. In Pakistan, 15% to 36% of sugarcane yield is lost due to stemborers, 10% to 20% by root-borers and 10% to 15% by top-borers [8]. Main objective of this study to prevent yield loss by making sugarcane resistant against stemborers and herbicides.

Materials and Methods

A gene cassette was designed that contains herbicides and stemborers resistant genes i.e. CEMB-*GTGene*, CEMB-*Cry*1Ac and CEMB-*Cry*2A under control of NOs terminator and maize ubiquitin promoter. These constructs pCEMB-SGTG and pCEMB-SC12 were introduced *via* electroporation into the *Agrobacterium* cells [9]. Colony PCR was performed to confirmed gene transformation *via* gene specific primers. 8 to 10 weeks old leaves of tobacco plants were co-cultured with *Agrobacterium* to induce *Agrobacterium* mediated transformation [10]. Expression of transgenes was indicated by histochemical detection of the GUS activity that was used as a reporter using agroinfiltrated leaves. Biolistic transformation method was used for transformation of transgene in 4 sugarcane varieties i.e. CPF-246, HSF-240, SPF-234 and SPF-213 [11]. During early transgenesis, transgene expression was determined by performing GUS assays on young shoots. Presence of transgenes was

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confirmed through PCR screening using CEMB-*GTGene*, CEMB-*Cry*1Ac and CEMB-*Cry*2A genes specific primers. Stable transgene integration was determined by performing southern blotting [12] on PCR positive transformants. CEMB-*GTGene*, CEMB-*Cry*1Ac and CEMB-*Cry*2A genes expression were determined through dipstick assays that were coated with the IgG monoclonal antibodies for each gene. These sticks were dipped in total proteins that were isolated from the fresh transgenic plants leaves [13]. ELISA was performed to quantify the transgene expressions. Toxicity effects of CEMB-*Cry*1Ac and CEMB-*Cry*2A endotoxins were determined by performing leaf biotoxicity assay on the leaves. CEMB-*GTGene* expression and activity was confirmed by spraying glyphosate on the transgenic plants. Comparison between different lines (control and transgenic) was done through statistical analysis (Dunnett's tests, LSD and ANOVA).

Results

Restriction of the CEMB-*GTGene*, CEMB-*Cry*1Ac and CEMB-*Cry*2A genes generated 1.4 kb, 1.8 kb and 1.9 kb fragments respectively which were then integrated into expression vector (pCAMBIA-1301). These constructs were introduced *via* electroporation into *Agrobacterium*. PCR analysis confirmed presence of transgenes. PCR positive transformants were subjected to agro-filtration using tobacco leaves in presence of GUS receptor. The expression of GUS was confirmed by bluish green color under fluorescent microscope. For sugarcane transformation and tissue culturing, CPF-246, HSF-240, SPF-234 and SPF-213 varieties of sugarcane were selected. To obtain maximum embryogenic calli from the selected varieties, 4 different combinations were used for callus induction media. Maximum embryogenic calli was observed in CPF-246 (100%) followed by SPF-213 (90%), SPF-234 (90%) and HSF-240 (81%). Plasmid constructs were then transferred to these varieties *via* biolistic methods. Total of 400 explants were used for transformation. On single selection media (kanamycin), 91% of CPF-246, 74% of SPF-234, 70% of SPF-213 and 45% of HSF-240 survived while on double selection media (glyphosate and kanamycin), 81% of CPF-246, 40% of SPF-234, 34% of SPF-213 and 29% of HSF-240 transformed calli survived. Then after it, GUS assay was performed to screen the transgenic putative plants. PCR, southern blotting, dipstick assay and ELISA was performed for transgenic plants at V_o, V_1 and V_2 generation. Leaf bio-assay was performed to determine the efficiency of CEMB-*GTGene*, CEMB-*Cry*1Ac and CEMB-*Cry*2A genes and 60% to 100% mortality rate *Chilo infuscattellus* was determined in transgenic leaves. Weed management was done by glyphosate spray assays and 70% to 76% of the transgenic plants were identified to be glyphosate resistant (3000 ml/Ha) in V_1 generation while 100% tolerant in V_2 generation (Figure 1).

Discussion

Main objective of crop production is to obtain high yields even for sugarcane [14]. Different viruses, drought stress, weeds and insects are the major constrains for sugarcane [15]. Present study aimed to control insects and weeds through genetic manipulation of sugarcanes. In this study, for maximum callus regeneration, an efficient procedure

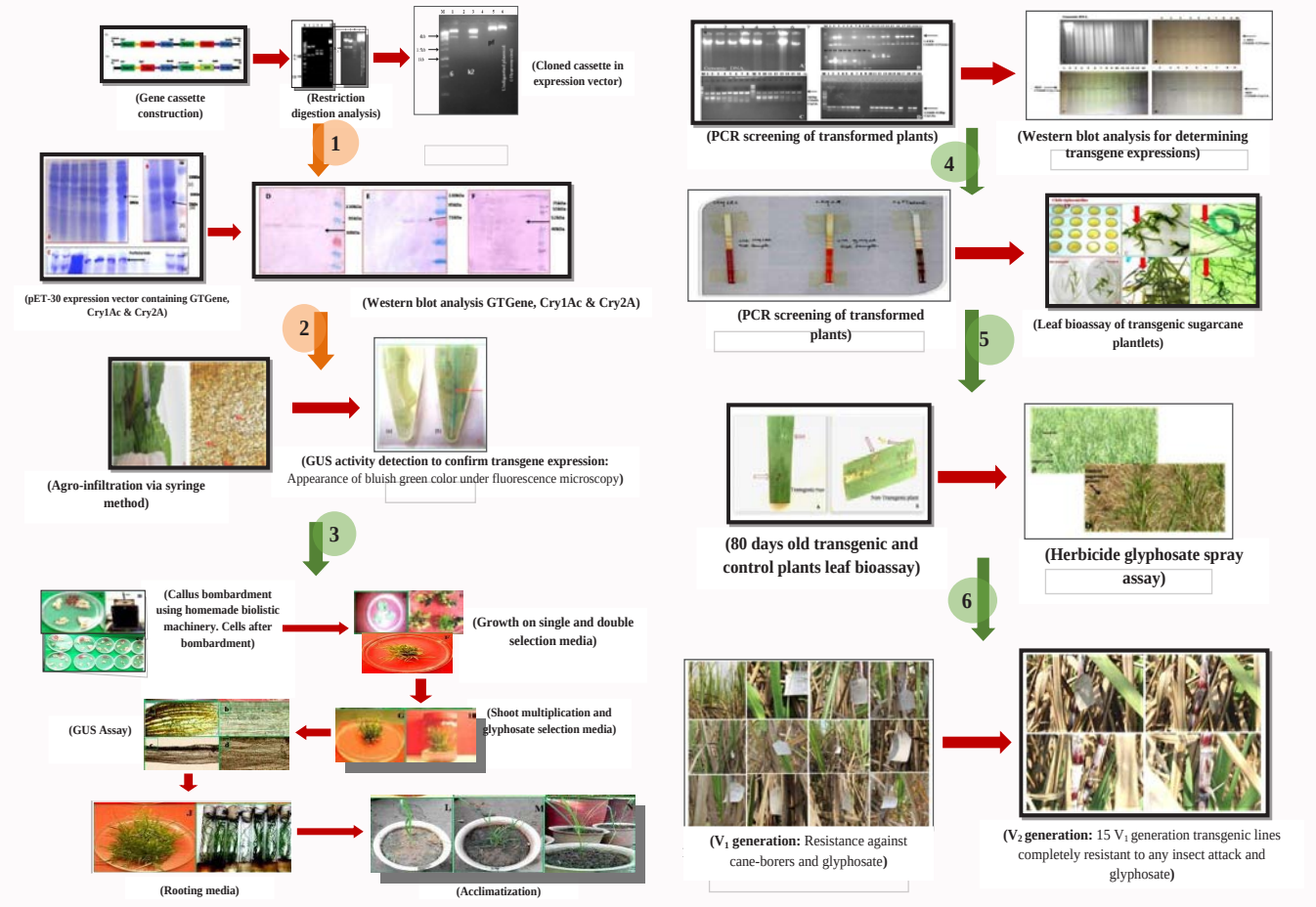

Figure 1:

was developed to instill tolerance against glyphosate and cane borers. For embryogenic callus formation, immature leaves were found to be excellent explants [16]. It basically strengthens the procedure for gene transformation in sugarcane [17]. From a callus inducing media with 2,4-D, embryogenic calli were obtained for all 4 varieties [18]. To enhance potential of embryogenic calli of sugarcane, it was supplemented with casein [19]. Tissue culture response was observed to critically screen all 4 varieties [20]. For genetic modification, varieties were selected on basis of regeneration response [21]. Studies have also disclosed that resistant against lepidopteran insects were best provided by Cry proteins [22]. Most commonly used herbicide for weed control is glyphosate which is a broad spectrum herbicide. One of the main drawbacks of using glyphosate is that along with weeds and herbs, it also stunts the plant growth thus affecting its yield [23]. It inhibits formation of EPSPS enzyme in shikimate pathway which leads to shikimate pathway being shut down. This inhibits the formation of 3 essential amino acids i.e. phenylalanine, tyrosine and tryptophan that humans can't synthesize and is required from plant source [24]. In this study, resistant against glyphosate and stemborers are provided by introducing CEMB-*GTGene*, CEMB-*Cry*1Ac and CEMB-*Cry*2A genes into the sugarcane varieties.

Conclusion

In this study, 100% mortality rates of *Chilo infuscatellus* have been found in CPF-246 variety showing that there was high resistant in transgenic sugarcanes against shoot borers and sufficient gene expression to fully resist target pests. Weed management was done by glyphosate spray assays and 70% to 76% of the transgenic plants were identified to be glyphosate resistant (3000 ml/Ha) in V_1-generation while 100% tolerant in V_2-generation. This study reported that after approval from biosafety committee, farmers can use this sugarcane variety as starting material for cost effective weeds and insect's control. More studies should be done to enhance stable *Bt.* toxin expression. Glyphosate resistant crops against 5000 mL/ha were recommended to be successful in controlling all sugarcane weeds.

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