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Studies on integration and expression of transgene (*cry1Ac*) in first clonal generation of Sugarcane (*Saccharum officinarum* L.)

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ABSTRACT

Sugarcane borers are the major biotic pest in sugarcane that accounts nearly 25-30% yield loses. Development of borer resistance in sugarcane through transgenic technology could be the best approach. Transgenic sugarcane for insect resistance was already developed. In the present investigation, we characterized first clonal generation of transgenics of sugarcane cv. 'CoJ 64' transformed with *cry1Ac* gene through molecular technique (Polymerase chain reaction and Reverse transcriptase polymerase chain reaction) and *in vitro* bioassay. Out of 17 transgenics plants of sugarcane 3 have shown the integration of transgene through PCR and 2 have shown expression of *cry1Ac* gene in plant through RT-PCR reaction. Plants were also screened by *in-vitro bioassay*. This study presents evidence that the *Bacillus thuringiensis* (*cry1Ac*) gene can be efficiently expressed in first clonal generation of sugarcane variety CoJ 64 and can resistance to the sugarcane borers.

Key words: Sugarcane, genetic transformation, *cry1Ac* gene, polymerase chain reaction, reverse transcriptase PCR, *in vitro* bioassay

Sugarcane (*Saccharum* spp., 2n= 100-205), a monocotyledon belonging to tribe Andropogoneae and family Poaceae, is widely grown in tropical and subtropical areas of the world. It is also one of the most efficient biomass crops and is being targeted for the integrated production of sugar and other high value industrial products (Nanato *et al.* 2001; Lakshmanan *et al.* 2005). Sugarcane (*Saccharum* species hybrids) occupies the largest cultivable area world wide amongst the sugar producing crops. Seventy eight per cent of the world's sugar is provided by sugarcane and India is the largest producer of sugar in the world, running neck to neck with Brazil, which sometimes excels India (Indian Sugar 2010). During the year 2011, sugarcane was grown on an area of 4.9 thousand ha and production was 339,168 million tones at the national level (Sachdevan *et al.* 2012).

Numerous biotic and abiotic factors influence the yield and quality of sugarcane. Among the biotic factors, sugarcane borers drastically reduce sugarcane yield and recovery in India or are a problem world wide. The crop is attacked by lepidopteran borers viz. early shoot borer (*Chilo infuscatellus*), top borer (*Scirpophaga excerptalis*), Gurdaspur borer (*Acigona steniellus*) and stalk borer (*Chilo auricilius*), right from germination to harvest. The extent of damage caused by borers is estimated from 20 percent (by top borer) to 100 per cent (by stalk borer). In Punjab also, all the sugarcane varieties including 'CoJ 64' are susceptible to borers during different stages of development. The commercially recommended sugarcane variety 'CoJ 64' is early maturing high sugar and highly responsive to tissue culture.

Developing a sugarcane cultivar with desirable traits through conventional breeding is a difficult task due to the complexities of the genetic makeup of the crop such as high polyploidy and heterozygosity (Rani *et al.* 2012). In this regard, development of insect resistance in sugarcane plants is the introduction of gene (s) that code for product (s) that interfere with insect development and mortality eg. soyabean proteinase inhibitors (Falco and Silva-Filho 2003) and cry proteins (Braga *et al.* 2003; Christy *et al.* 2009; Weng *et al.* 2011) serve as a promising strategy. *Cry* genes from *Bacillus thuringiensis* are proven to confer resistance against lepidopteran insects.

The most commonly employed methods of gene transfer include particle gun bombardment and *Agrobacterium*-mediated genetic transformation. Particle gun method holds immense potential for carrying out genetic enhancement as it is a genotype independent method and can be used to co-bombarded more than one gene simultaneously (Chen *et al.* 1998). Transformation of monocot crop plants has been achieved primarily by particle gun bombardment (Bower and Birch 1992). Sugarcane was first transformed by Bower and Birch (1992) with the particle gun using neomycin phosphotransferase II (*nptII*) gene for selection. Subsequently production of stable transgenic sugarcane has been reported by several other workers (Arencibia *et al.* 1995; Gallo-Meagher and Irvine 1996; Fitch *et al.* 1996). The first insect resistant transgenic sugarcane was reported in 1997 using

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electrophoresis gene transfer method (Weng *et al.* 2011). During the year 2011, a total of 17 transgenic plants (PCR positive) developed carrying *cryIAc* gene (for borer resistance) of sugarcane cv. CoJ 64 through particle gun. These were propagated conventionally and Ist clonal generation (T_1) was raised during the year 2012.

Once transformation experiments are done the next step is to analyze the putative transgenic plants using PCR (Polymerase Chain Reaction), RT-PCR (Reverse transcription polymerase chain reaction), Southern blotting, Western blotting and insect bioassay. Many workers have used these techniques for molecular analysis of putative transgenic of sugarcane plants (Braga *et al.* 2003; Zi-Zhang *et al.* 2005; Ai-Qin Wang *et al.* 2009; Christy *et al.* 2009). In this investigation we employed the molecular techniques viz. Polymerase chain reaction, Reverse transcription PCR and *in vitro* insect bioassay to examine the integration and expression of the transgene in first clonal generation of PCR-positive transgenic sugarcane cultivar 'CoJ 64' carrying *cryIAc* gene.

MATERIALS AND METHODS

PCR analysis of T_1 generation of transgenic sugarcane

Plant genomic DNA was isolated from 200 mg young leaf tissue of each of the 17 T_1 plants of sugarcane cv.'CoJ 64' using cetyl trimethyl ammonium bromide method (Doyle and Doyle 1990). A non-transformed plant of the same age raised through tissue culture and propagated conventionally during the year 2012 was used as control. About 50 ng of genomic DNA (3µl) was used as template for 20 µl reaction. PCR mix contained 4µl of 10X PCR buffer (promega), 4 µl of dNTP mix (100 µM, promega), *cryIAc* gene specific primers containing each of 1µl of 1µM of forward (5'-TGGAGAACGCATTGAAACCG-3') and reverse (5'-TGTTGCTGAATCCGGAACGC-3'), 1.2 µl of $MgCl_2$ (25mM, promega), 1.0 unit (0.3 µl) of Taq DNA polymerase and 5.5 µl of sterile distilled water.

PCR conditions used for amplification were initiated with initial denaturation at 94°C for 4 min, followed by denaturation 94°C for 1 min, annealing at 54°C for 1 min, extension for 1 min at 72°C, final extension at 72°C for 7 mins. The amplified DNA was analysed through electrophoresis on 1.0% agrose gel.

RNA isolation and Reverse Transcription (RT) - PCR analysis

The expression of *cryIAc* gene at transcriptional level was checked by RT-PCR (Reverse Transcriptase Polymerase Chain Reaction). For expression of the transgene at transcriptional level, cDNA synthesis by RT PCR and amplification of cDNA by using specific designed primers were carryout out at same PCR profile.

First of all, the total RNA was isolated from the young leaves of PCR positive transformed and control (non-transformed) plants of sugarcane by Fast RNA Green kit. The cDNA synthesized from total RNA by BioRad-iScript cDNA synthesis kit. The synthesized cDNA amplified by Polymerase Chain Reaction by using *cryIAc* gene specific designed primers.

Total RNA isolated from the transgenic leaves was used as template for cDNA synthesis. Each 20µl reaction, added 5X iScript reaction mixture (4µl), Reverse transcriptase enzyme (1 µl), Nuclease free water 13 µl and RNA template (50ng) 2 µl.

PCR conditions used for synthesis of cDNA were initiated with initial 25°C for 5 min, followed by 42°C for 30 min and 85°C for 5 min.

Bioassay of transgenic sugarcane plants for insect resistance

For the *in vitro* insect resistance bioassay, RT PCR confirmed transgenic sugarcane plants of first clonal generation were used. Experiment was conducted both on the transgenics and non transgenic sugarcane (control) plants. Neonate larvae of *chilo auricilius* were inoculated or released inside the stalk portion of transgenic and non transgenic sugarcane plants. The larvae were being weighed after every 2 days interval upto one week.

RESULTS

Molecular analysis of first clonal generation of transgenic plants of sugarcane

First clonal generation of 17 PCR positive T_0 plants of sugarcane cv. 'CoJ 64' were subjected to PCR analysis. PCR analysis was done using *cryIAc* gene specific primers. Out of 17 plants, 3 showed the amplification of *cryIAc* (927 bp) gene there by indicating the presence of the gene in the genome of T_1 generation of sugarcane (Fig 1a.). Rest of 14 plants showed the transient gene expression. During PCR analysis non transformed plants were included as negative control to check the proper amplification.

Reverse transcription polymerase chain reaction

Based on PCR data, the cDNA of 3 PCR positive T_1 transgenic plants of sugarcane were amplified by gene specific (*cryIAc* gene) primers through polymerase chain reaction. Out of three PCR positive transgenic plants (confirmed by PCR), two showed expression of transgene (*cryIAc*) by RT-PCR (Fig 1b).

Insect bioassay of transgenic plants

The non transformed plants, the larvae of stalk borer (*Chilo auricilius*) grew and developed normally; feeding on the stalk portion and on the transgenic plants also the larvae grew and developed normally during the first few days of the infestation. Larvae weight of one transgenics (-0.0315) was significantly less than as compared to other transgenics plants and control plants of 'CoJ 64' after feeding. Data showed negative relationship between cry protein and larvae weight. The neonate larvae did not die but grew more slowly compared with controls. The fresh weight of insect larva was decreased after few day intervals (Table 1.). Expression of transgene at translational level is slow.

Table 1 Bioassays of transgenic sugarcane lines (carrying *cryIAc* gene) and control using stalk borer (*chilo auricilus*) larvae

Plant number	Stage of larva	Weight (g) at 0 day	Weight (g) after 7 days	Change in weight from 0 to after 7 day (g)
200	4th instar	0.0695	0.0691	-0.0004
62b (Tiller no. 1)	4th instar	0.0642	0.0862	0.0220
62b (Tiller no. 2)	Before pupa	0.0619	Died due to injury	died
62 b (Tiller no. 3)	Before pupa	0.0854	0.0539	-0.0315
Control	Before pupa	0.0691	0.0796	0.0105

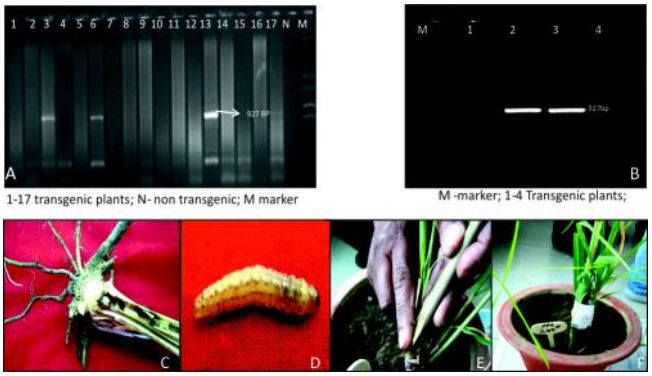

Fig 1 Molecular analysis and in vitro bioassay of first clonal generation of transgenic sugarcane cv. 'CoJ 64'

A) PCR analysis of first clonal generation of transgenic plants of sugarcane cv. CoJ64
B) cDNA synthesis by RT-PCR showing the expression of *cryIAc* gene at transcriptional level in the transgenic plants of sugarcane cv. CoJ64
C) Early shoot borer infected portion of sugarcane
D) View of early shoot borer
E) Transgenic sugarcane plants being infected with borer
F) *In vitro* insect bioassay in transgenic sugarcane plants

DISCUSSION

The objective of the present investigation was to characterize first clonal generation of PCR-positive sugarcane cv. 'CoJ 64' through molecular analysis and *in vitro* insect bioassay.

Advances in both transformation and heterologous gene expression techniques in plants allowed the generations of insect resistant plants which express cry proteins from Bt. Up to date different versions of natural Bt genes and chimeric constructions have applied to develop self pesticide transgenic plants from several species (Vazquez *et al.* 1995).

Sugarcane is highly amenable to genetic transformation and regeneration via either biolistic bombardment (Bower and Birch 1992) or *Agrobacterium* mediated (Arencibia *et al.* 1999; Elliott *et al.* 1998) technique. Traditional DNA delivery methods used in plant transformation proved to be practical in sugarcane, namely electroporation (Arencibia *et al.* 1995), *Agrobacterium*-mediated (Arencibia *et al.* 1998; Elliott *et al.* 1998; Ennquez-obrego ne *et al.* 1998; Manickavasagam *et al.* 2004) and particle bombardment (Bower *et al.* 1996; Bower and Birch 1992; Gallo-Meagher and Irvine 1996; Ingelbrecht *et al.* 1999; Snyman *et al.* 2006).

Sugarcane transgenics were initially produced through particle bombardment. Early attempts to develop transgenic sugarcane with insecticidal genes involved introduction of modified *cryIAc* gene and the transgenics showed resistance to the borer *Elasmopalpus lignosellus* (Fitch *et al.* 1996; Weng *et al.* 2006). Subsequently genes coding for *cryIAb* (Vazquez *et al.* 1995; Arencibia *et al.* 1997; Arvinth *et al.* 2010) were used to develop resistance to different borers. In attempts with other toxins, a proteinase inhibitor from *Nicotiana alata* engineered into sugar cane produced marked antibiosis to cane grubs (Nutt *et al.* 1999) and soybean proteinase inhibitors showed resistance to borers (Falco and Silva-Filho 2003). Fitch *et al.* (1996) obtained transformed sugarcane with Bt gene for resistance to the lesser cornstalk borer. Insect bioassay indicate the LCB larvae that fed on some calli or leaves weighed less and showed higher mortality than those fed on non-transgenic tissues. Arencibia *et al.* (1997) introduced *cryIAb* gene coding the active region of the *Bacillus thuringinensis* endotoxin in sugarcane plants. Genetic transformation was accomplished by electroporation of intact cells. The levels of recombinant toxin were established and biological activity tests were performed against neonate sugarcane borer (*Diatraea saccharalis* F.) larvae. Transgenic sugarcane plants showed significant larvicidal activity despite the low expression of *cryIAb*. Braga *et al.* (2003) also analysed transgenics through molecular characterization and insect feeding bioassays. They introduced *cryIAb* gene into sugarcane plants. This present study evidence that the *B. thuringiensis cryIAb* gene can be efficiently expressed in sugarcane plants over an extended growing period and can confer resistance to the sugarcane borer under field conditions. Christy *et al.* (2009) checked the integration expression and functions of the transgene (synthetic gene coding aprotinin) through southern, western and insect bioassay respectively. In *in-vivo* bioassay studies of larvae top borer *Scirpophaga excerptalis* walker (Lepidoptera: pyralidae) feed on transgenics showed significant reduction in larval weight which indicated impairment of their development. Wua *et al.* (2009) studied susceptibility of *cryIAb* resistant and susceptible sugarcane borer to four *bacillus thuringiensis* toxins. Neonate of *D. Saccharalis* were assayed on a meridic diet containing one of the four cry proteins larval mortality, body weight and number of surviving loarvae that

did not gain significant weight (0.1 mg/larva) were recorded after 7 days. *cryIAa* was the most toxic protein against both insect strains, followed in decreasing potency by *cryIA105*, *cryIAc* and *cry2Ab*. Weng *et al.* (2011) reported transformation of sugarcane with *cryIAc* gene through particle bombardment. The integration sites and expression pattern of the transgene were determined by southern, northern and western blot analyses. In green house plant assay about 62% of the transgenic lines exhibited excellent resistance to heavy infestation by stem borers.

The present study showed that integration and expression of transgene in first clonal generation of sugarcane but toxin showed a lower level of field tolerance to the borer.

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