RESEARCH ARTICLE

Expression patterns of *cp4-epsps* gene in diverse transgenic *Saccharum officinarum* L. genotypes

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Abstract Glyphosate, a functional analogue of phosphoenolpyruvate (PEP), blocks the shikimate pathway by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) through interference with the conversion of (shikimate-3-phosphate) S3P and PEP to 5-enolpyruvylshikimate-3-phosphate (EPSP) and subsequently leads to plant death. This metabolic pathway possesses great potential to be used for development of herbicide resistant transgenic crops and here in this study, we wanted to check the expression potential of *CP4-EPSPS* gene in various sugarcane genotypes. A synthetic version of *CP4-EPSPS* gene synthesized commercially, cloned in pGreen0029 vector, was transformed into

regenerable embryogenic calli of three different sugarcane cultivars HSF-240, S2003US-778 and S2003US-114 using biolistic gene transfer approach for comparative transcriptional studies. Transgenic lines screened by PCR analysis were subjected to Southern hybridization for checking transgene integration patterns. All the tested lines were found to contain multiple (3–6) insert copies. Putative transgenic plants produced the CP4-EPSPS protein which was detected using immunoblot analysis. The *CP4-EPSPS* transcript expression detected by qRT-PCR was found to vary from genotype to genotype and is being reported first time. In vitro glyphosate assay showed that transformed plants were conferring herbicide tolerance. It is concluded that different cultivars of sugarcane give variable expression of the same transgene and reasons for this phenomenon needs to be investigated.

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Introduction

A chloroplast-bound and nuclear-encoded enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), has central role in the shikimate pathway for the biosynthesis of aromatic amino acids and secondary metabolites like phytoalexins, lignin, auxins etc. (Tzin and Galili 2010). Glyphosate (*N*-phosphonomethyl glycine)—a broad-spectrum, systemic and potent herbicide—is a functional analogue of phosphoenolpyruvate (PEP) and binds in ternary complex of glyphosate, shikimate-3-phosphate (S3P) and EPSPS, and blocks the shikimate pathway by inhibiting the enzyme EPSPS through interference with the conversion of S3P and PEP to

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5-enolpyruvylshikimate-3-phosphate (EPSP) and subsequently leads to plant death (Pollegioni et al. 2011). EPSP synthases have been classified into two classes; type I EPSP synthases found in bacteria and plants are completely sensitive to glyphosate, while type II EPSP synthases identified in bacteria usually are completely glyphosate tolerant and have high affinity for PEP (Cao et al. 2012). Consideration of EPSPS for the development of herbicide resistant crops was made after the identification of recognition site for glyphosate action in 1980. Earlier attempts to produce glyphosate resistant plants through over expression of their indigenous EPSPS failed to yield any successful transgenic event (Dill 2005). Later on, it was reported that mutation in EPSPS may lead to its enhanced expression which might be sufficient enough for conferring resistance in plants against glyphosate. After research comprising of three decades, only a few transgenic herbicide traits encoded by EPSPS type II genes from *Agrobacterium* sp. CP4 and mutants of type I EPSPS from maize or *E. coli* transformed into commercial crops are available to the farmers.

Sugarcane species belong to perennial grasses of the genus *Saccharum* and modern cultivars are hybrids of *S. officinarum* and *S. spontaneum* with high level of polyploidy ($2n=100\text{--}130$ chromosomes). There is a huge interest in engineering sugarcane varieties through molecular and biotechnological approaches, because conventional breeding is extremely difficult to employ in genetic improvement of sugarcane clones due to their poor fertility and complex polyploid–aneuploid nature. In addition to this, retrieval of elite sugarcane cultivars with required traits through conventional breeding is an extremely time consuming process (Butterfield et al. 2001). However, tendency of suppressed transgene expression in sugarcane is a big hurdle in the exploitation of useful traits in sugarcane. Recently, various synthetic, codon optimized genes were shown to generate higher transgene expression in sugarcane (Kinkema et al. 2014). Proportion of GC content and related codon bias make a vital impact on transgene expression and this difference in transgene expression might be due to less availability of rare codons encoded by specific tRNAs. These rare codons disturb the translation process by destabilizing mRNA transcripts by exposing them to RNA degrading machinery. Designing of synthetic genes with higher GC contents using redundancy of the genetic code can increase the translational efficiency and eliminate the AT rich sequence which proves deleterious for mRNA stability (Welch et al. 2011). Here in this study, we designed *EPSPS* gene according to plant preferred codon usage by avoiding RNA instability motifs in order to check the transcription potential of transgene in various genetically transformed *Saccharum officinarum* L. genotypes.

Materials and methods

Designing and optimization of *CP4-EPSPS* gene

Nucleotide sequence encoding CP4-EPSPS was retrieved from database. Gene was designed after codon optimization according to plant preferred codons (<http://www.genious.com>) by paying considerations to constraints such as regulatory elements and cryptic splice sites, selenocysteine incorporation, strings of A and T, rare codons, palindromes, polyadenylation signals, and possibility of introns RNA methylation signals, stable mRNA structures, direct repeats etc. using the algorithms as described by Gustafsson et al. (2004). *CP4-EPSPS* gene synthesized with CaMV promoter and E9 yeast cofactor terminator was restricted from pUC57 vector using *EcoRI* and *HinDIII* and cloned in pGreen0029 expression vector at the same restriction sites. The resultant plasmid (pGEPC) was prepared using maxiprep kit (QIAGEN) following manufacturer's instructions.

Transformation and regeneration of transgenic sugarcane

Two advanced lines (S-2003US114, S2003US778) and one commercial cultivar (HSF-240) acquired from Sugarcane Research Institute, Faisalabad, Pakistan were used in this study. Embryogenic calli were bombarded with plasmid pGEPC. Regeneration of calli was carried out according to methods described by Bower et al. (1996). Putative transgenic plants were shifted to shooting medium. All the regenerated plantlets (120 plants of cultivar US2003S-778, 38 plants of cultivar US2003S-114 and 78 plants of HSF-240) were transferred to rooting medium in the jars containing glyphosate 1% (v/v). The glyphosate tolerant plants, with well-developed roots were shifted to pots containing peat moss and placed in growth room at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ under a 16 h photoperiod for acclimatization purposes. Plants which successfully acclimatized were transferred to large pots containing soil and placed in glasshouse at $27 \pm 5\text{ }^{\circ}\text{C}$ under natural light till maturity.

Molecular confirmation of putative sugarcane transgenic plants

Genomic DNA was extracted from meristematic leaves of young sugarcane plants using DNAeasy kit according to manufacturer's instructions. Verification of putative transgenic plants was carried out using PCR analysis with gene specific EPSPS primers, PF5-5'/CCTCACCTCCTGCGAG ACG GA 3', and PR5-5'/CGCCGCTACCGGATGCAG ATT 3'. Transgene integration into the genome of

randomly selected lines was carried out through Southern hybridization using ECL Direct Labeling and Detection System (GE Healthcare, Germany). About 20 µg genomic DNA extracted from wild-type and transgenic sugarcane plant lines was digested with *EcoRI*, and was immobilized on Hybond-N⁺ membrane. Amplified fragment of 625 bp using primers from *EPSPS* CaMV promoter region (CaMV-F TGAGGATACAACCTTCAGAGA, *EPSPS*6-R TCCATTTCCAACGCCGTCAAT) was used for preparing the probe following manufacturer's instructions.

Gene expression analysis using qRT-PCR

Total RNA was extracted from newly emerged leaves of putative transgenic sugarcane plants using trizol method followed by treatment with DNaseI. First strand cDNA synthesis was performed using commercial cDNA synthesis kit (Applied Biosystems, USA). *EPSPS*1 (5'-TGGG TTTGGTTGGTGTGTTT-3'; 5'-AAGTTATGGGAGTGGG AG-3') and *GAPDH* (5'-CACGGCCACTGGAAG CA-3', and 5'-TCCTCAGGGTTCTGATGCC-3') specific primers amplifying 180 bp and 150 bp fragments, respectively were used for Real time quantitative PCR (qRT-PCR) analyses. For relative quantification of *CP4-EPSPS* mRNA, wild-type cDNA was used as a negative control. The reaction mixture consisted of 0.4 µL of each primer, cDNA 2 µL (prepared from 1 µg of total RNA), 12.5 µL platinum SYBR Green Supermix (Thermo-Fisher Scientific, Waltham, MA, USA) with final volume reaction of 25 µL. PCR reactions were performed in Real-Time PCR Detection System (Applied Biosystems, USA) in 96-well plates. The samples were run in triplicate. PCR machine was programmed at 94 °C for 5 min for 1 cycle followed by 40 cycles consisting of 20 s at 94 °C, 20 s at 60 °C and 20 s at 72 °C, followed by final extension of 10 min at 72 °C.

Immuno-strip analysis of EPSPS protein

Expression of CP4-EPSPS protein was assessed in the transgenic and non-transgenic control lines of sugarcane using Immuno-strip test (QuickStix Envirologix, Portland, OR, USA). The crude protein was extracted from the young sugarcane leaves using extraction buffer according to the manufacturer's instructions. Immuno-strips were immersed in the extracted samples for 10 min and samples were scored as negative or positive for transgene expression depending upon the absence or presence of visible test line.

Statistical analysis

Statistical analyses were performed using the software PASW Statistics 18 (formerly SPSS Statistics; SPSS Inc., Chicago, IL, USA [<http://www.spss.com.hk/statistics/>]). All the mean values were compared using Least Significant Difference (LSD) test after performing Analyses of Variance (ANOVA).

Results

Designing of CP4-EPSPS gene

Average GC contents in *Saccharum officinarum* is 55% (www.kazusa.or.jp/) while *CP4-EPSPS* gene coding sequence from *Agrobacterium tumefaciens* cp4 strain has 41% GC contents. We harnessed the redundancy of genetic code for designing synthetic version of *CP4-EPSPS* gene encoding sequence, and to eliminate strings of A and T, rare codons, palindromes and polyadenylation signals. The synthetic version of *CP4-EPSPS* contains GC content of 45.5%. The CP4-EPSPS gene was cloned in pGreen0029 at *EcoRI* and *HindIII* restriction sites.

Verification of putative sugarcane transgenic plants

The expression cassette cloned in plasmid pGEPC (Fig. 1) was transformed into *S. officinarum* plants using biolistic mediated approach (Fig. 2).

All the putative sugarcane transgenic plants were analyzed by PCR to confirm the integration of CP4-EPSPS transgene. A total of 78 lines out of the 236 putative transgenic plants showed amplified product of 565 bp with gene specific primers, suggesting that the CP4-EPSPS gene carried by expression vector was integrated into the plant genome successfully (Fig. 3a). Moreover, the integration of transgene was further confirmed through Southern hybridization which showed the presence of multiple inserts in majority of the tested lines of all the cultivars (Fig. 3b).

Gene expression analysis using qRT-PCR

Total RNA was isolated from all of the PCR positive transgenic lines using Trizol method. About 1 µg of RNA was reverse transcribed to cDNA after DNase treatment. All the cDNA samples were screened for confirmation of transcriptional activity of transgene using semi-quantitative PCR. Total of 32 lines showed the transcription activity. Quantitative real-time PCR (qRT-PCR) was used to analyze expression levels of *CP4-EPSPS* gene in the randomly selected transgenic lines of each cultivar and to evaluate

Fig. 1 The expression cassette, consisting of a Cauliflower mosaic virus (CaMV) promoter, the *CP4-EPSPS* gene and an E-9 terminator from yeast cofactor, was cloned as a *EcoRI-HindIII* fragment into the binary vector pGreen0029 to produce pGEPC plasmid for plant transformation

Fig. 2 Various stages involved in the transformation of synthetic *CP4-EPSPS* gene in sugarcane using biolistic approach; **a** embryogenic calli placed on MS media; **b** non-transformed calli placed on MS media supplemented with geneticin 40 mg/L; **c**, **d** shoots

emerging from calli placed on MS shooting media; **e** shoots placed on rooting medium; **f** Non-transformed plants failed to develop roots on medium containing 1% glyphosate; **g** plants with well-developed root system; **h** putative transgenic plants shifted to pots

any possible relationship of gene expression and cultivar genotype. Meristematic parts of plant leaves grown in the

green house were used for analyses. Relative gene expression levels were found to differ many fold within the

Fig. 3 **a** Agarose gel electrophoresis of PCR products for verification of the integration of *cp4-epsps* gene in genomic DNA of sugarcane. PCR fragments of a 565 bp amplified using gene specific EPSPS primers; lane M, 1 kbp DNA ladder; lane 1, negative (water) control; lane 2, negative (non-transformed sugarcane DNA) control; lane 3, empty well; lane 4, positive control (pG-EPC plasmid); lanes 5–12, PCR amplified products from genomic DNA of representative putative transgenic plants. **b** Southern hybridization analysis of representative sugarcane plants transformed with *cp4-epsps* gene.

Genomic DNA (20 µg) from wild type and transgenic plants, and pG-EPC plasmid was digested with *EcoRI* and resolved on 0.8% agarose gel electrophoresis for 14 h. The resolved gel was shifted on to the nylon membrane. CaMV-EPSPS 569 bp labelled fragment was probed with the blot. Lane 1 shows the linearized plasmid used as positive control and lane 2 shows the untransformed wild type plant while 6 independent transgenic plants are shown in lanes 3–7 respectively. All the lanes show multiple copy integration (3–6)

lines of one cultivar (Fig. 4) and also in the lines from one cultivar to another.

The highest relative expression levels of *CP4-EPSPS* gene were found in the lines GTT-3, GTT-92, GTT-6 of HSF-240 cultivar, while lowest expression was detected in the line GTT-66, and GTT-54 of US2003S-114 cultivar. Higher gene expression was recorded in transgenic lines of HSF-240 cultivar followed by US2003S-778 lines. Least

transgene expression was detected in transgenic lines of US2003S-114 cultivar (Fig. 5).

Immuno-strip analysis of CP4-EPSPS protein

The transgenic plants expressing CP4-EPSPS protein confers resistance to glyphosate. The Immuno-strip test was carried out to analyze the CP4-EPSPS gene expression

Fig. 4 Accumulation of CP4-EPSPS gene transcripts in representative transgenic and wild type sugarcane plants. Total RNA was extracted from leaves of transgenic and wild type sugarcane plants, reverse-transcribed to cDNA and subjected to qRT-PCR analyses. Relative mRNA transcript levels of *CP4-EPSPS* gene were calculated with respect to the transcripts level of *GAPDH* gene. The mean

transcript levels were compared by LSD after performing ANOVA. Bars are the mean standard error calculated from three biological replicates and three technical replicates. Significant differences ($P = 0.05$) are indicated by different letters. X axis shows different transgenic lines, and Y axis shows normalized expression of transgene

Fig. 5 Average relative mRNA transcript levels of CP4-EPSPS gene in three different sugarcane cultivars. The mean mRNA values were compared by LSD after performing ANOVA. Significant differences

($P = 0.05$) are indicated by different alphabets. X-axis shows different transgenic lines, and Y-axis shows normalized expression of transgene

in transgenic plants. Transgenic lines were assayed at the development stage of ~ 20 internode. The tested plants showed specific bands, suggesting that mRNA synthesized by plants was being translated into protein, while there was

no protein detected in the wild-type control plant samples (Fig. 6).

Discussion

Codon optimization of foreign gene sequences has been a remarkable way to enhance transgene expression in many plant species. Modification in sequences of foreign transgene in a way to mimic to indigenous coding sequences of host plant can be maneuvered to avoid triggering of host plant's interactions against foreign gene sequences. Codon usage modification increases translational efficiency if stalling of rare codons directed at the ribosome is prevented. Modern design rules followed during the course of this study, also considered avoiding known RNA destabilizing motifs and polyadenylation signals. Increasing GC contents may also eliminate unknown AT rich RNA destabilizing sequences by chance, which suggest that increase in gene expression might be due to increased transcript stability through sequence modifications other than translational efficiency only (Chou and Moyle 2014).

An efficient biolistic mediated transformation procedure with some variations was followed to transform all three different cultivars (US2003S-114, HSF-240, and US2003S-778) with good callus transformation efficiency, which is in accordance with previous reports by Vickers et al. (2005) and Noguera et al. (2015). The qRT-PCR analyses were carried out to examine transgene expression patterns in meristematic tissues in sugarcane genotypes HSF-240, S2003US-778, and S2003US-114 using qRT-PCR. These polyploid cultivars are widely planted all over the world. The expression levels of transgene varied among three genotypes. Genotypes S2003US-778 and S2003US-114 were found to be relatively transcription recalcitrant in comparison to HSF-240 genotype, which generally offered

Fig. 6 CP4-EPSPS protein analysis of representative transgenic sugarcane lines by immunoblot analysis. Lanes 1–8, protein bands from crushed leaf samples of representative transgenic sugarcane lines; lane 9, negative control (crushed leaf samples from non-transformed sugarcane)

appreciable transgene expression levels in all the tested lines.

There was more variability in transgene expression among plants from different cultivars, although plant-to-plant expression differences appeared to be relatively smaller. These results are in accordance with the other findings (Adamczyk and Meredith 2004) which show higher variability in Cry1Ac expression among various cotton cultivars and relatively lower differences among lines of same cultivar. These comparative transcriptomics results are very interesting and being reported for the first time in sugarcane genotypes. There can be various citable reasons behind variable gene expression, for example, interaction of host gene and transgene may facilitate aberrant RNAs and/or cRNA to confer posttranscriptional gene silencing. Gene silencing varies with the ploidy level, higher the ploidy level higher is the gene silencing. Higher transgene methylation, transcription repression rates, and lower transgene expression have been reported in 4n plants in comparison to 2n Arabidopsis plant. Initially, higher transcript abundance was thought to be associated with transgene mRNA degradation, and later, it was found that increased transcription was not always the case in silenced plants. The presence of inverted repeats (IR) at transgene locus plays a crucial role in silencing (Finn et al. 2011).

All of the transgenic lines examined contained multiple copies of transgene inserts. Multiple transgene inserts could be attributed with higher translation rates (Altpeter et al. 2005). However, the correlation between multiple transgene copy number and gene expression may not be straightforward to explain as a number of previous studies declined the correlation between higher gene expression and multiple transgene insertions in sugarcane (Jackson et al. 2013).

The variable mean expression results of EPSPS gene among transgenic lines of all of the three cultivars are also consistent with the previous finding (Adamczyk and Sumerford 2001) which showed variable Cry1Ac δ -endotoxin expression among different transgenic cotton cultivars. They found that Bt toxin level reduction in cotton cultivars was associated with reduction in Cry1Ac transcript levels. Decline in Cry1Ac expression in cotton cultivars was associated with reduction in the levels of mRNA production (Finnegan et al. 1998). Continuous presence of retrotransposons in between the genes, and copy number changes associated with microsatellites has their profound impact on alteration of DNA contents in crop plants. Thus, different varieties of same crop vary in genome sizes for example, various maize varieties differ by 42% and chili varieties differ by 25% of their DNA contents. Inward and outward jumping of transposable elements can alter the gene expression or serve as sites of chromosomal

arrangements or breakage, and such changes have the potential to alter the DNA and protein profiles of the plants (Wessler 2001). Genes change their location in the genome as shown by the comparison of closely related grass species (Lai et al. 2004). Different varieties of the same species do not necessarily comprise of same number of genes due to variation in number of transposon and retrotransposons (Fu and Dooner 2002) and thus plants can form completely new genes with novel functions by incorporating sequences from other genes (Jiang et al. 2004). Factors influencing level of transgene expression among various cultivars are yet to be fully known, but gene insertion site and genetic background have been found to be associated (Sachs et al. 1998). The transgene expression in genetically modified crops is significantly affected by environmental factors such as water stress, nitrogen deficiency, light and temperature (Dong and Li 2007). Similarly factors such as rainfall, soil characteristics, biotic stresses, and appropriate farming management have direct or indirect impact on crop performance and may affect the expression of transgenes. These factors, inherent in environment and varieties, make the difference between optimal or suboptimal performance of crop cultivars. Leaf tissue with low chlorophyll content does not express transgene fully which suggests that mRNA transcription and translation is influenced by photosynthesis regulating factors. Increased rates of nitrogen fertilizers in various cotton cultivars had been reported to enhance the expression of Bt genes due to production of leaves with higher chlorophyll concentration (Pettigrew and Adamczyk 2006). Therefore, managing transgenic cultivars properly to maintain their overall vigor may be mandatory for benefiting the full transgenic genetic potential. Variable transgene expression among various cultivars may make development of synthetic genes more challenging and evaluation of transgenic varieties would require advanced resistance monitoring strategies at larger scale. Investigation at genetic, molecular, and physiological levels can further help explore the reasons behind differential expression of transgenes.

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