Molecular cloning and functional analysis of the promoter of γ -Tocopherol Methyl Transferase (γ -TMT) gene of soybean (*Glycine max*)

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Abstract

γ -Tocopherol methyl transferase (γ -TMT) (EC 2.1.1.95) is the key enzyme of the tocopherol biosynthetic pathway that determines the α -tocopherol concentration in plants. The overexpression of γ -TMT has been a successful approach for α -tocopherol enrichment of most plants including soybean. The typical soybean varieties are rich in γ -tocopherol (constitutes nearly 65–70% of its total seed tocopherol pool), while α -tocopherol, the biologically most active form among all tocopherols, constitutes only 10% of the total tocopherol content. The identification of soybean varieties that have seed α -tocopherol as high as >20% of the total tocopherols has shifted attention towards the breeding based approach for α -tocopherol enrichment of this crop. Previous research on this aspect suggests that polymorphisms in γ -TMT promoter might be associated with the high α -tocopherol concentration of some soybean varieties. To understand the molecular basis of genetic variation for α -tocopherol concentration in Indian varieties of soybean we cloned the 1.4 kb upstream promoter region of γ -TMT from a high α -tocopherol containing soybean variety (Bragg) as well as from a low α -tocopherol containing variety (DS 2706). Cloning of each of these promoters in pORE R2 vector having GUS reporter gene and the subsequent GUS assay revealed a slightly high promoter activity of Bragg γ -TMT as compared to DS 2706 γ -TMT. On promoter sequence analysis, no sequence polymorphisms were observed in the core promoter region of this gene. However, seven single nucleotide polymorphisms (SNPs) were observed outside the core promoter region. Further study based on deletion construct analysis of this promoter will elucidate the significance of these SNPs in influencing the activity of γ -TMT promoter and the α -tocopherol concentration.

Keywords γ -TMT · α -Tocopherol · Promoter · Soybean · Polymorphism · SNPs

Introduction

Tocopherols are amphipathic molecules that exist in four different forms, i.e., alpha (α)-, beta (β)-, gamma (γ)-, and delta (δ)-tocopherols that differ on the basis of number and position of methyl groups on their chromanol head group

(Kamal-Eldin and Appelqvist 1996). The role of tocopherols in preventing lipid oxidation is known since 1931 (Cummings and Mattill 1931) and the health benefits of tocopherols are the outcome of their being a remarkable antioxidant in lipophilic environment. Daily intake of vitamin E in excess of the Recommended Dietary Allowance (400 IU/250 mg of RRR- α -tocopherol) is associated with decreased risk of cardiovascular diseases, improved immune function, and slow progression of a number of degenerative human diseases (Buring and Hennekens 1997; Tangney 1997; Bramley et al. 2000). The biological activities of α -, β -, γ - and δ -tocopherols vary with the number and position of methyl groups on the chromanol ring (Bramley et al. 2000; Fryer 1992). Among all isomers of tocopherols, (R, R, R) α -tocopherol has the highest vitamin E activity for humans and animals (Sheppard et al. 1993; Bramley et al.

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2000) due to the specificity of hepatic tocopherol binding protein for this particular tocopherol. Vitamin E activities of β -, γ - and δ -tocopherols are 50, 10 and 3% equivalent to that of α -tocopherol activity (Kamal-Eldin and Appelqvist 1996; Sheppard et al. 1993; Shintani and DellaPenna 1998). Therefore, α -tocopherol is the most important tocopherol for human health.

The tocopherols are synthesized exclusively by photosynthetic organisms, and therefore, plants, particularly oilseeds, are the major source to meet our dietary requirement of α -tocopherol. Approximately 85% of commercial vitamin E is chemically synthesized (Subramaniam et al. 2001) and it is a racemic mixture of eight stereoisomers whose biological activity ranges from 50 to 74% of that of natural α -tocopherol (Clemente and Cahoon 2009). Therefore, considering the demand for natural α -tocopherol the α -tocopherol enrichment of plants is of much importance. Soybean oil accounts for 30% of the worldwide oil consumption. With an average of 100 mg of tocopherols per 100 g of oil it is considered as one of the rich source of tocopherols (Eitenmiller 1997). However, despite being a rich source of tocopherols, the vitamin E activity of soybean oil is relatively low due to its low α - to γ -tocopherol ratio (Eitenmiller 1997; Grusak and DellaPenna 1999). α -Tocopherol, the biologically most relevant form of tocopherols accounts for only 10% of total tocopherols present in soybean oil, while the less bioactive γ -tocopherol accounts for nearly 60–65% of the total tocopherols present in it (Tan 1989). Therefore, the manipulation of tocopherol biosynthetic pathway in soybean to convert the less bioactive γ -tocopherol to the biologically more relevant α -tocopherol is of special interest for human health.

Breeding for enhancement of α -tocopherol content of soybean is dependent on the genotypic variability for α -tocopherol concentration. Soybean varieties have been identified that have α -tocopherol concentration as high as 20–30% (Ujiie et al. 2005). QTL analysis in soybean has revealed that γ -tocopherol methyl transferase (γ -TMT) is the candidate gene which determines the concentration of α -tocopherol (Dwiyanti et al. 2007, 2011, 2016). γ -TMT is the last enzyme of the tocopherol biosynthetic pathway which methylates γ - and δ -tocopherols to produce α - and β -tocopherols, respectively (d' Harlingue and Camara 1985; Hofius and Sonnewald 2003; Koch et al. 2003). Comparison of full length gene sequence of Japanese soybean varieties (KAS, Dobrudza 14 Pancevo, Dobrogeance, Ichihime and Toyokomachi) has revealed that the 5' upstream region of γ -TMT in the high and low α -tocopherol containing varieties has significant differences that could be responsible for the higher expression level of this enzyme in high α -tocopherol containing varieties and vice versa.

To further explore the molecular basis of genotypic variability of α -tocopherol concentration in soybean we cloned

the 1.4 kb promoter region from two Indian soybean varieties Bragg and DS 2706 that have contrasting α -tocopherol concentration (10.48 and 1.36 $\mu\text{g/g}$, respectively) and compared their nucleotide sequence and various *cis*-acting elements. To test the hypothesis that the genotypic variation in α -tocopherol concentration of Bragg and DS2706 varieties could be due to the differences in their promoter elements, we compared the promoter sequence of γ -TMT in both the varieties, identified the various *cis* elements present in this promoter and studied the activity of γ -TMT promoter in these two varieties by performing histochemical GUS assay in transgenic *Arabidopsis thaliana* leaves.

Materials and methods

Biological materials and chemicals

Two soybean varieties (Bragg and DS2706) that have contrasting α -tocopherol content were previously identified by screening of 80 soybean varieties for their α -tocopherol concentration through high performance liquid chromatography (Vinutha et al. 2015). The seeds of these varieties were procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi. This screening identified two varieties, Bragg which is a high α -tocopherol containing variety of soybean (10.48 $\mu\text{g/g}$) and DS 2706, a low α -tocopherol containing variety (1.36 $\mu\text{g/g}$). pGEM[®]-T Easy vector (Promega, USA) and pORE R2 vector (obtained from Division of Biochemistry, I.A.R.I, New Delhi) were used to create recombinant plasmids. *E. coli* (DH5 α) was purchased from New England Biolabs (England). *A. thaliana* wild-type plants' ecotype Col-0 was used in this study. The seeds were germinated as per the protocol described by Zhang et al. (2006). The plants were grown at 22 ± 2 °C temperature, 18 h day and 6 h night condition at National Phytotron Facility, New Delhi. We purchased chemicals from the following sources: restriction enzymes from New England Biolabs (England), Dream Taq DNA Polymerase, T4 DNA Ligase, dNTPs, O'GeneRuler 1 kb DNA Ladder and Gene Ruler 1 kb DNA ladder from Thermo Fisher scientific (USA), isopropyl- β -D-thiogalactopyranoside (IPTG), X-gal, ampicillin and kanamycin from Sigma (USA); rifampicin from Genetix Biotech Pvt. Ltd., agarose, Luria-Bertani Broth (LB) and Luria- Bertani Agar (LA) from HiMedia (India); TRI reagent, T4 DNA Ligase, O'GeneRuler 1 kb DNA Ladder and Gene Ruler 1 kb DNA ladder from Thermo Fisher scientific (USA).

Estimation of tocopherols in soybean genotypes

The tocopherols were extracted from soybean flour using *n*-hexane and estimation was done by HPLC (Rani et al. 2007).

C18 silica column was used as the stationary phase. Separation was accomplished using 100% methanol at a flow rate of 1.0 ml/min for 20 min. Oven temperature was maintained at 40 °C. Tocopherols were detected at 295 nm using a UV detector.

Genomic DNA isolation and PCR

Seeds of Bragg and DS2706 variety of soybean were germinated on petriplates and genomic DNA was isolated from their hypocotyls according to a CTAB-based protocol (Doyle and Doyle 1987). The transcription start site of this gene was already known. The 1.4 kb upstream region from the transcription start site of γ -TMT gene was selected from the DNA sequence of *Glycine max* chromosome 9 (Chr09: 44340570...44342002) available at NCBI database. Using the sequence, the following primer pairs were designed F1: 5'CCGCTCGAGGCCTATTGGCCACCTTGTCAC3' and R1: 5'CGCGGATCCACACCTCCTCTCTCCTTGCTCTCTT3'. *Xho*I site of forward primer (F1) and *Bam*HI site of reverse primer (R1) are underlined. Using genomic DNA as a template, the 1.4 kb promoter region was amplified from both the varieties using these primers. The 50 μ l reaction mixture for PCR consisted of 100 ng genomic DNA, 0.4 μ M of each primer, 0.2 mM of dNTP (each), 10 $\times$ Dream Taq buffer and 1.25 U of Dream Taq DNA polymerase and nuclease free water was used to make up the volume to 50 μ l. PCR was carried out in following steps: (1) initial denaturation at 94 °C for 5 min, (2) denaturation at 94 °C for 1 min, annealing at 57 °C for 60 s, extension at 72 °C for 5 min, (3) 35 cycles of step 2, (4) final extension at 72 °C for 10 min. The amplified promoter region from both varieties, i.e., Bragg and DS2706 was analyzed on agarose gel and thereafter PCR purified using Qia-gens PCR purification kit. The same promoter region from the both Bragg and DS 2706 variety of soybean was also amplified using Phusion polymerase which is a high-fidelity DNA polymerase. The same set of primers F1 and R1 that were used for amplification of the promoter with Dream Taq DNA polymerase were used for amplification with Phusion polymerase as well. The 50 μ l reaction mixture for PCR amplification consisted of 100 ng genomic DNA from soybean, 0.4 μ M of each primer, 0.2 mM of dNTP (each), 1X Phusion HF buffer and 1.0 IU of Phusion polymerase and volume was made to 50 μ l with nuclease free water. The same thermal cycling programme that was used for amplification of the promoter with Dream Taq DNA polymerase was also used here.

Designing of promoter constructs (γ -TMT promoter-pGEM[®]-T Easy and γ -TMT promoter-pORE R2)

The purified Dream Taq DNA polymerase amplified promoter DNA from both varieties was initially cloned in

pGEM[®]-T Easy vector system. The ligated product was transformed in the host *E. coli* (DH 5 α). Plasmid was isolated from transformed colonies using alkaline lysis method and digested overnight with *Xho*I and *Bam*HI (Green and Sambrook 2012). The restriction positive plasmid was sequenced from GCC Biotech by Sanger sequencing. To clone the promoter in pORE R2 binary vector, the 1.4 kb promoter region of both the varieties that was amplified with Phusion polymerase and PCR purified as well as the pORE R2 plasmid were restricted overnight with *Xho*I and *Bam*HI restriction enzymes. Separate ligation reactions were kept for sticky end ligation of each promoter sequence with pORE R2 vector at 4 °C. The ligated products were then transformed into host and the recombinant colonies were confirmed on LA media supplemented with kanamycin (35 μ g/ml). Plasmid was isolated from recombinant colonies using alkaline lysis method and digested overnight with *Xho*I and *Bam*HI (Green and Sambrook 2012). The restriction positive plasmids were sequenced from GCC Biotech. Ltd. by Sanger sequencing. The *Gm* γ -TMT promoter sequence thus derived from both varieties of *G. max* was assembled and submitted to BankIt NCBI. The in silico analysis of *cis*-regulatory elements in the promoter sequences from both genotypes was done using PLANTCARE database (Lescot et al. 2002).

Transformation in *Arabidopsis thaliana* and screening of transformants

Each construct, i.e., *Gm* γ -TMT (Bragg) promoter: pORE R2 and *Gm* γ -TMT (DS2706) promoter: pORE R2 was mobilized into *Agrobacterium tumefaciens* strain EHA 105 by the freeze–thaw procedure. The transformed *A. tumefaciens* colonies were confirmed on LA medium supplemented with kanamycin (35 mg/l) and rifampicin (50 mg/l). The recombinant colonies were also confirmed by colony PCR using γ -TMT promoter specific primers F1 and R1 mentioned earlier. Four-week-old *A. thaliana* plants having good number of unopened inflorescence were selected for *Agrobacterium*-mediated transformation by floral dip method (Zhang et al. 2006). The floral dip suspension consisted of 5% sucrose solution containing 0.02% (v/v) Silwet L-77 and resuspended *Agrobacterium* cells carrying the promoter to be transferred. The transformed plants were kept in dark for 12 h and thereafter grown under normal conditions. On maturation of siliques, the seeds were collected and dried at room temperature. The seeds thus harvested were sterilized and subjected to selection on MS media supplemented with kanamycin (30 μ g/ml) as selection agent. These plates were kept in *Arabidopsis* growth chamber. On attainment of four leaf stage the *Arabidopsis* plants which sustained the kanamycin selection and developed roots were transferred from kanamycin containing selection media to pots containing soilrite mix (mixture of horticulture grade expanded perlite,

Irish Peat moss and exfoliated vermiculite in equal ratio, i.e., 1/3:1/3:1/3) at National Phytotron Facility, New Delhi. After 4 weeks from the date of transplantation, genomic DNA was isolated from leaves of these transformed plants by the CTAB method (Doyle and Doyle 1987) and transformation was confirmed by means of a PCR reaction using γ -TMT promoter specific primers F1 and R1. The 20 μ l reaction mixture for PCR consisted of 100 ng genomic DNA from transformed *Arabidopsis* plants, 0.4 μ M of each primer, 0.2 mM dNTP (each), 10X Dream Taq buffer and 1.25 U of Dream Taq DNA polymerase and nuclease free water to 20 μ l. The PCR programme used was the same as that used for amplification of γ -TMT promoter from soybean genomic DNA with Dream Taq polymerase. In another PCR reaction, the transformed *A. thaliana* plants were confirmed using GUS specific primers F2: 5'CAACGAACTGAAGTGGCA GA3' and R2: 5'AGAGGTTAAAGCCGAGAGCA3'. The 20 μ l reaction mixture for PCR screening with GUS specific primers consisted of 100 ng genomic DNA from transformed *Arabidopsis* plants, 0.4 μ M of each primer, 0.2 mM dNTP (each), 10X Dream Taq buffer and 1.25 U of Dream Taq DNA polymerase and nuclease free water to 20 μ l. PCR was carried out in following steps: (1) initial denaturation at 94 °C for 5 min, (2) denaturation at 94 °C for 1 min, annealing at 54 °C for 45 s, extension at 72 °C for 1 min, (3) 35 cycles of step 2, (4) final extension at 72 °C for 10 min. The leaves of the plants that were found positive in PCR screening for both γ -TMT promoter as well as GUS gene (*uid A*) were used for histochemical GUS staining.

Histochemical GUS staining

The leaves of PCR positive plants were tested for GUS assay. Fresh leaves were taken and kept in GUS staining solution (50 mM sodium phosphate buffer pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 1% Triton X 100, 20% methanol, 2 mM X-Gluc) and incubated overnight at 37 °C (Jefferson et al. 1987). After staining, the leaves were treated with 70% ethanol to remove chlorophyll. The stained section of leaves was then examined and captured under compound light microscope.

Quantitative expression analysis of GUS gene

A quantitative assay of GUS activity was performed by qRT PCR analysis of GUS (*uidA*) gene. RNA was isolated from the leaves of GUS positive *Arabidopsis* plants using TRI reagent and it was quantified. Its purity was evaluated by 260/280 absorbance ratio and by gel electrophoresis. First strand cDNA was synthesized using 1 μ g of total RNA according to the protocol of Thermo Fisher scientific (USA) RevertAid first strand cDNA synthesis kit (Cat. no. K1622). qRT PCR was carried out in Real-Time PCR Detection

System (Bio-Rad, USA). The actin gene of *A. thaliana* was used as the reference gene for assaying of GUS gene expression. The reaction was carried out in a volume of 20 μ l using 100 ng cDNA, kappa SYBR Green fast qPCR kit master mix (2x) and respective primer pairs for GUS (GF: 5'TCACAG CCAAAGCCAGACA3' and GR: 5'GCGAGGTACGGT AGGAGTTG3') and Actin (AF: 5'ACAGCAGAGCGGGAA ATTGT3' and AR: 5'TCCATTCCCACAAACGAGGG3'). The reactions were carried out in triplicates and the average Ct value was calculated.

Results and discussion

Isolation and cloning of γ -TMT promoter sequence from high and low α -tocopherol containing genotypes of soybean

The 1432 bp γ -TMT promoter sequence upstream of the transcription start site (TSS) was amplified from both the varieties Bragg as well as DS 2706 (Figs. 1, 2). On the basis of results obtained by sequencing of γ -TMT- pGEM[®]-T Easy and γ -TMT- pORE R2 clones the final sequence of γ -TMT promoter was assembled from both DS 2706 and Bragg variety of soybean and submitted in NCBI Gene Bank under the accession no. KX371096 and KT033495, respectively. So far, there are very few reports on the cloning and characterization of γ -TMT promoter. The few reports available are from *Arabidopsis* (Zhou et al. 2006) and soybean (Dwiyanti et al. 2011).

Analysis of cis-regulatory elements in γ -TMT promoter sequence

The 1432 bp promoter sequence of γ -TMT from Bragg as well as DS 2706 variety of soybean was analyzed for the presence of cis-regulatory elements by PlantCARE database (Lescot et al. 2002). The core promoter elements TATA box (TATAAAA) and CAAT box (CAATT) were identified at –28 and –45 positions from the transcriptional start site (TSS) of *Gm* γ -TMT in both the varieties (Fig. 3). The canonical sequence of core promoter elements, i.e., TATA box and CAAT box was found to be conserved in the promoter of both the varieties (Fig. 3). This finding differs from a previous study on genetic variation in γ -TMT gene of some soybean varieties which reports differences in the canonical CAAT box sequence as well as the Myb binding site among the promoter sequence of high and low α -tocopherol containing varieties of soybean (Dwiyanti et al. 2011). Outside the core promoter region, seven single nucleotide polymorphisms (SNPs) were found in the promoter sequence of Bragg and DS2706 (Fig. 3) at positions –91, –537, –699, –835, –983, –1197, and –1338 from the TSS.

Fig. 1 Cloning of *Gm* γ -TMT promoter from DS 2706 variety of soybean in pORE R2 vector. **a** pORE R2 vector restricted with *Bam*HI and *Xho*I (lanes 1 and 2), *M*: 1 kb DNA ladder, **b** colony PCR of DS 2706 γ -TMT promoter transformed in DH5 α cells (lanes 1 and 2), *M* 1 kb DNA ladder; **c** DS 2706 γ -TMT promoter restricted from recombinant pORE R2 plasmid using *Bam*HI and *Xho*I (lanes 1, 2 and 3), *M* 1 kb DNA ladder

Fig. 2 Cloning of *Gm* γ -TMT promoter from Bragg variety of soybean in pORE R2 vector. **a** pORE R2 vector restricted with *Bam*HI and *Xho*I (lanes 1 and 2), *M*: 1 kb DNA ladder; **b** colony PCR of Bragg γ -TMT promoter transformed in DH5 α cells (lanes 1, 2, 3, and

4), *M*: 1 kb DNA ladder; **c** Bragg γ -TMT promoter restricted from recombinant pORE R2 plasmid using *Bam*HI and *Xho*I (lanes 1, 2, 3, 4 and 5), *M*: 1 kb DNA ladder

The in silico analysis of γ -TMT promoter from both DS 2706 and Bragg variety of soybean revealed the presence of many *cis*-regulatory elements involved in light responsiveness which includes ACE (AAAACGTTTA), box 4 (ATTAAT), G box (CACGTA) and I box (ATGATATGA) (Table 1). An increased accumulation of α -tocopherol under high light stress was observed in *A. thaliana* and other crops (Collakova and DellaPenna 2003; Porfirova et al. 2002; Szymańska and Kruk 2010). The other major *cis* elements observed in γ -TMT promoter from both DS 2706 and Bragg variety of soybean includes ABRE (TACGTG), a *cis*-regulatory element responsive to abscisic acid and HSE (AAAAAATTTC), a *cis*-regulatory element responsive to heat stress. The presence of ABA related *cis* elements in the γ -TMT promoter is consistent with previous reports, where ABA is reported to induce the expression of enzymes related to oxidative stress (You and Chan 2015). ABA treatment is also known to increase the concentration of tocopherols in maize (Jiang and Zhang 2001).

Promoter activity of γ -TMT from Bragg and DS 2706 variety of soybean in transgenic *Arabidopsis thaliana*

To study the promoter activity of γ -TMT from both Bragg and DS2706 we infected the *A. thaliana* plants with *Agrobacterium* cells transformed with *Gm* γ -TMT (Bragg) promoter: pORE R2 and *Gm* γ -TMT (DS2706) promoter: pORE R2 plasmids, respectively, by floral dip method according to the protocol given by Zhang et al. 2006. Plants raised from the seeds harvested from the transformed *A. thaliana* plants were subjected to kanamycin selection (Fig. 4) and at later stage to PCR mediated selection for the presence of γ -TMT promoter (Fig. 5) and GUS gene. GUS staining of the leaves of transgenic (T1) *A. thaliana* plants showed comparatively higher expression of GUS gene (*uid A*) in the high α -tocopherol containing Bragg variety, while low expression of GUS was observed in the low α -tocopherol containing DS 2706 variety (Fig. 6) which proves a slightly high γ -TMT

Fig. 3 Predicted core promoter elements and single nucleotide polymorphisms in the promoter region of Bragg and DS2706 variety of soybean

promoter activity in Bragg as compared to DS 2706. To quantify the difference in the expression of GUS reporter gene in these varieties a quantitative real-time expression analysis was done. The relative expression of GUS (*uid*

A) gene in Bragg variety was found to be 1.8-fold higher than its expression in DS2706 variety (Fig. 7).

Because the core promoter region is found to be conserved in both the promoters, therefore, for the correct identification of the promoter elements that are responsible

Table 1 Regulatory *cis* elements identified in the promoter region of DS2706 and Bragg variety of soybean based on PlantCARE database

<i>cis</i> element	Motif	Putative function
ABRE	TACGTG	Involved in the abscisic acid responsiveness
ACE	AAAACGTTTA	Involved in light responsiveness
Box 4	ATTAAT	Involved in light responsiveness
CAAT box	CCAAT	Common <i>cis</i> -acting element in promoter and enhancer regions
G box	CACGTA	Involved in light responsiveness
HSE	AAAAAATTC	Involved in heat stress responsiveness
I box	ATGATATGA	Part of a light responsive element
MBSI	AAAAAAC(G/C)GTTA	MYB binding site involved in flavonoid biosynthetic genes regulation
Skn-1_motif	GTCAT	<i>cis</i> -acting regulatory element required for endosperm expression
TATA box	TATAAAT	Core promoter element around – 30 of transcription start required for basal expression
TC-rich repeats	ATTTTCTTCA	Involved in defense and stress responsiveness
TCA element	CCATCTTTTT	Involved in salicylic acid responsiveness

Fig. 4 Selection of transformed *Arabidopsis thaliana* seeds. **a** *Arabidopsis thaliana* seeds grown on MS media without kanamycin (control); **b** *Arabidopsis thaliana* seeds obtained from plants transformed with Bragg γ -TMT promoter-pORE R2 and DS 2706 γ -TMT

promoter-pORE R2 construct, grown on MS media with kanamycin. Only Kanamycin resistant transgenic seedlings retain the dark green colour, while the non-transformed ones are of light green colour

Fig. 5 PCR screening of transformed *Arabidopsis thaliana* plants using γ -TMT promoter specific primers. **a** PCR screening of *Arabidopsis thaliana* plants (transformed with DS 2706 γ -TMT promoter-pORE R2 construct) using γ -TMT promoter specific primers; **b** PCR screening of *Arabidopsis thaliana* plants (transformed with Bragg γ -TMT promoter-pORE R2 construct) using γ -TMT promoter specific primers **Fig. 6** GUS staining of leaves from *Arabidopsis thaliana* plants. **a** GUS stained leaves of *Arabidopsis thaliana* plant (transformed with Bragg γ -TMT promoter-pORE R2 construct); **b** GUS stained leaves of *Arabidopsis thaliana* plant (transformed with DS 2706 γ -TMT promoter-pORE R2 construct); **c** GUS stained leaves of untransformed *Arabidopsis thaliana* plant (control)

for the higher promoter activity of Bragg variety a deletion construct analysis is required which will identify the major *cis* elements that influence the promoter activity. Further

Fig. 7 qRT PCR analysis of relative GUS expression in the leaves of transgenic *Arabidopsis thaliana* plants having Bragg γ -TMT promoter driven *uid A* gene and those having DS 2706 γ -TMT promoter driven *uid A* gene

exploration of this research by means of deletion construct analysis can also reveal which of the identified SNPs have influence on the promoter activity of *Gm* γ -TMT.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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