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Expression analysis of γ-tocopherol methyl transferase genes and α-tocopherol content in developing seeds of soybean [*Glycine max* (L.) Merr.]

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Soybean, *Glycine max* (L.) Merr., besides being rich source of protein and oil, is a good source health promoting substances *viz*., isoflavones, anthocyanins and tocopherols, the latter are composed of four isoforms α-, β-, γ- and δ-tocopherols. Due to the potent antioxidant property and nutraceutical relevance of α-tocopherol, increasing its levels endogenously by modulating γ-tocopherol methyl transferase (*γ-TMT*) gene expression has gained significant attention in recent years. Despite having γ-tocopherol as the predominant form, its conversion into highly active α-tocopherol is the limiting step in soybean seeds; the step which is catalyzed by γ-tocopherol methyl transferase enzyme. Based on the differences in amino acid sequence at NH2-domain, the *γ-TMT* is classified as *γ-TMT1, γ-TMT2* and *γ-TMT3*. Expression of these *γ-TMT* genes was studied in developing soybean seeds of two contrasting genotypes with respect to α-tocopherol content, namely Bragg ('high') and DS-2706 ('low'). Among *γ-TMT*, significantly higher (P <0.05) expression (2-4.5 fold) of *γ-TMT3* was observed in Bragg genotype than in DS-2706 at 50 days after flowering (DAF). A positive correlation was observed between *γ-TMT3* gene expression and α-tocopherol accumulation (14 µg/g seed) in Bragg at 50 DAF than in DS-2706. The study may prove useful in greater understanding of temporal differences in the α-tocopherol accumulation and *γ-TMT3* gene expression in seeds and the distinct roles of *γ-TMT* genes in soybean.

Keywords: *Glycine max*, Soybean seed, *γ-TMT* gene, α-Tocopherol

Soybean, *Glycine max* (L.) Merr., is one of the most widely cultivated crops of the world, mainly for their high protein (40%) and oil (21%) content[1]. The unique nutritional profile of soybean is a major reason for its cultivation and consumption in varied forms such as tofu, natto, miso, soy sauce, soy flour, soy protein or defatted soy meal and soymilk[2,3]. Besides, soybean also contains a number of health promoting compounds, such as isoflavones, anthocyanins, saponins and vitamin E[4-9].

Tocopherols, commonly termed as vitamin-E, possess potent antioxidant properties and are synthesized only by photosynthetic organisms i.e., higher plants and green algae. Out of four (α-, β-, γ- and δ-) isoforms, α-tocopherol exhibits the highest activity in humans. Because of the potent free radical scavenging properties, α-tocopherol is considered as the most powerful antioxidant[10]. In plants, tocopherols are involved in intracellular signaling, protection of fatty acids in stored seeds and improving the membrane stability[11], whereas in humans, vit-E leads to decreased risk for cardiovascular disease, cancer, and caducity, aids in immune function, and prevents or slows down many degenerative diseases[12-14].

Of the various isoforms, α-tocopherol is usually the predominant in leaves, while δ- and γ-tocopherols are the major forms in seeds[15]. The variations in tocopherol isoforms are also detected in seeds, such as soybean, rapeseed (*Brassica napus*) and Arabidopsis (*Arabidopsis thaliana*) with most of the tocopherols present in the form of γ- or δ-tocopherol; however, in sunflower (*Helianthus annuus*) and safflower (*Carthamus tinctorius*) seeds, α-tocopherol comprises more than 95% of the total tocopherol content[16,17]. Wide variations in α-tocopherol content (µg/100 mg seed powder) and concentration (ratio of α- and total tocopherols) have been reported in crops, such as maize (0.9-6.5 µg 100 mg^{-1}), sunflower (>95% in wild type and <10% in mutants), safflower (>85% in wild type and <15% in mutants), rapeseed and in the model plant Arabidopsis (α/γ-tocopherol ratio ranged from 0.54 to 1.70)[18-20], while it is only 4-10% in soybean oil[21].

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The majority of tocopherols in soybean seeds are found in the cotyledon (92.8%), but small quantities are also detected in the seed coat (5.4%) and embryonic axis (1.8%)[22]. Soybean seeds typically contain 4-10% α-, 1-3% β-, 60-66% γ- and 24-29% δ-tocopherols[23].

Despite having γ-tocopherol as the predominant form, its conversion into highly active α-tocopherol is a limiting step in soybean seeds; the step which is catalyzed by an enzyme γ-tocopherol methyl transferase by utilizing S-adenosyl methionine (SAM) as its cofactor[24] is utilized to elevate vit-E levels in the seeds. Due to nutraceutical relevance of α-tocopherol, increasing its levels endogenously by modulating *γ-TMT* expression has gained significant attention in the recent years. So far, the *γ-TMT* has been cloned from several species and overexpressed in many of the important crops[25-28]. Significant increase in α-tocopherol content in soybean seed[29] and lettuce[30] has been demonstrated using the *γ-TMT* gene from the model plant *Arabidopsis thaliana*[31]. The overexpression of *γ-TMT* in Arabidopsis seeds is reported the oil composition in favor of α-tocopherol[31]. Higher expression of *γ-TMT3* and its close correlation with high α-tocopherol content is reported in developing seeds of soybean[32].

Although India ranks fifth in the world in area and production of soybean[1], commensurate research in the direction of molecular and genetic basis of tocopherol content in soybean is still in infancy. However, over two hundred soybean germplasm collections, including wild types and their derivatives have been screened for different tocopherols (data not published). In this study, we have investigated the expression of *γ-TMT* genes and their correlation with α-tocopherol content in two contrasting genotypes differing widely in α-tocopherol content, namely Bragg (high α-tocopherol content) and DS-2706 (low α-tocopherol content). The study may help in understanding the molecular basis of variation in α-tocopherol levels in soybean seeds.

Materials and Methods

Plant material

Two widely contrasting soybean genotypes with respect to α-tocopherol content, namely Bragg ('high') and DS-2706 ('low') [germplasm collections were provided by the Division of Genetics, I.A.R.I, New Delhi] were used in the study. Seeds collected at 30, 40 and 50 days after flowering (DAF) were immediately frozen in liquid nitrogen and stored at -80°C for further investigation.

RNA isolation and cDNA first strand synthesis

RNA extraction was performed by the trizol reagent (Invitrogen, USA) as described by the manufacturer. RNA was dissolved in DEPC treated water and intact RNA bands were checked on 1.5% agarose gel. The quantity and quality (in terms of protein and DNA contamination) of isolated RNA was analyzed by Nanodrop spectrophotometer (Thermo Scientific, USA).The first strand cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific-India) following manufacturer's instructions and the resultant cDNA was used as template for both RT-PCR and qRT-PCR reactions carried out in our study.

Primers designing and real-time quantitative polymerase chain reaction (qRT-PCR) assay

All the primers (Table 1) were designed from the batch primer3 tool available at the website (www.http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi). Gene-specific primers were designed from *Glycine max γ-TMT* sequences [Glyma09g35680.1 (*γ-TMT3*), Glyma12g01680.1 (*γ-TMT1*) and Glyma12g01690.1 (*γ-TMT2*)] given in the phytozome (www.phytozome.net) database and the primers for EF1α reference (endogenous) gene was designed from the *Glycine max* EF1αsequences (XM_003553245.1) from NCBI.

Differential expression of mRNA was quantified by qRT PCR and analyzed using CFX96 Touch™ Real-Time PCR detection system of Bio-Rad, India. PCR reactions were performed in 96-well plates

Table 1—Primers of *γ-TMT* genes and reference gene (*EF1α*)

Name of primer	Gene ID		Primer sequence
γ-TMT1	*Glyma09g35680.1**	F	AGAAGGAGGGGA AACTTCA
		R	GAAACAGAGGCAA AGCGAAG
γ-TMT2	*Glyma12g01680.1**	F	GGATCCCAACAAT CTCATGC
		R	TGTCATCCTTCTTC GGCTTC
γ-TMT3	*Glyma12g01690.1**	F	GTGGAGCAGAAAG CAGCAG
		R	CGCGATGATCAGA AACAGAA
EF1α	XM_003553245.1†	F	ACAGAGGCTCTTC
		R	CAGGTGAATCGCC TGTCAATCTTGGTC

Note: * denotes the name of the gene identity number taken from Phytozome database; †denotes gene id taken from NCBI database. F, Forward; R, Reverse

Biosystems using SYBR Green detection chemistry to detect dsDNA synthesis. The PCR reaction mixture was 20 ngcDNA, 10 µL 2X fast sybr green master mix, 0.2 µL forward primer (10 pmol), 0.2 µL reverse primer (10 pmol) and 7.6 µL nuclease-free water in a total volume of 20 µL. PCR reaction was performed as follows: an initial denaturation step at 95°C for 20s, followed by 35 cycles of 95°C for 6 s, 58°C for 1 min and 65°C for 1 min, followed by a final extension step at 72°C for 7 min. Non-template controls were included for each primer pair and each PCR reaction was performed in triplicate. PCR products were separated on 1% agarose gels and bands were visualized under UV illumination.

Total tocopherol extraction and estimation by HPLC

Total tocopherols were extracted from seeds at 30, 40 and 50 DAF and analyzed by reverse-phase high performance liquid chromatography (HPLC), following the procedure described by Dwiyanti *et al*.[32] with major modifications. 500 mg of seed sample was weighed and ground into a fine powder using liquid nitrogen. After adding 2.5 mL of hexane, it was vortexed for proper mixing. The sample was sonicated at 4°C for 10 min, incubated at 4°C for 30 min and centrifuged at 10000 rpm for 10 min at 4°C; the resultant supernatant was subjected to hexane evaporation using rotary evaporator, the remaining residue/oil was dissolved in 200 µL isopropanol. Analysis was performed in an HPLC system (Shimadzu, model-SCL-10AVP, Japan) with an Inertsil ODS-3 reverse-phase column (3.0 mm × 250 mm, Shimadzu corporation pvt. Ltd., Japan). Column temperature was maintained at 40°C and separation was performed under isocratic condition for 20 min. Solvent A was acetonitrile, solvent B was methanol and the ratio of solvent A to solvent B was 50:50 (v/v) and a flow rate of 0.5 mL/min was maintained; tocopherols were detected at 295 nm.

Cloning and sequence analysis of γ-TMT coding sequences

Partial coding sequences of γ-TMT1, γ-TMT2 and γ-TMT3 were amplified by using the following PCR conditions: initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing temperature for 50 s, 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were gel eluted and ligated into pGEM-t easy vector (Promega Biotech India Pvt. Ltd.) and sequenced by (SciGenom Sequencing Services, Kochi, India). Sequence data were assembled and analyzed by BioEdit v7.2.5 and BlastX programme was used to identify the similar sequences available in NCBI database. The plant species which showed >60% homology (Table 2) were selected for motif and conserved domain analysis.

Motif analysis was done by using MEME web server (http://meme.nbcr.net) hosted by the National Biomedical Computation Resource[33]. The search parameters were: motif width: 6-50 amino acids with a maximum 3 motifs for discovery. A motif was considered significant when present in most of the members grouped together in the phylogeny or similar to motif identified in the tocopherol methyl transferases of other plant species. The phylogenetic tree was generated for the γ-TMT protein sequences given in Table 2 using neighbor–joining (NJ) method implemented in MEGA 4.0 software. The alignment of γ-TMT sequences was performed with phylogeny with collapse branches having branch support value smaller than 50% and the NJ tree was bootstrapped by 1000 bootstrap trials to confirm the robustness of the branches.

Results and Discussion

Functional analysis of γ-TMT genes

Based on the difference in the N-terminal region of γ-TMT proteins, Dwiyanti *et al*.[32] classified γ-TMT genes into *γ-TMT1*, *γ-TMT2* and *γ-TMT3*. On the basis of soybean genome information in the phytozome (www.phytozome.net) database, it is found that γ-TMT1

Table 2—γ-Tocopherolmethyltransferase protein sequences retrieved from the NCBI database (http://www.ncbi.nih.gov.)

Organism	Sequences identity compared with *Glycine max*	Accession no.
Arabidopsis thaliana	76%	NP176677.1
Artemisia Sphaerocephala	76%	ACS34775.1
Brassica napus	75%	ACD03287.1
Brassica oleracea	76%	AAO13806.1
Carthamus oxyacanthus	79%	AFO70131.1
Cicer arietinum	86%	XP004498827.1
Gossypium hirsutum	78%	ABE41798.1
Helianthus annuus	77%	ABB52800.1
Lotus japonicus	82%	AAY52459.1
Morus notabilis	80%	EXB29127.1
Perilla frutescens	79%	AFP68180.1
Prunus mume	78%	XP008241299.1
Saccharum hyb. Cv. R570	75%	AGT16736.1
Solanum pennellii	76%	AD224710.1
Solanum tuberosum	77%	NP001275191.1
Theobroma cacao	76%	XP007029706.1
Triticum aestivum	76%	CAI77219.2
Zea mays	74%	AGF92809.1

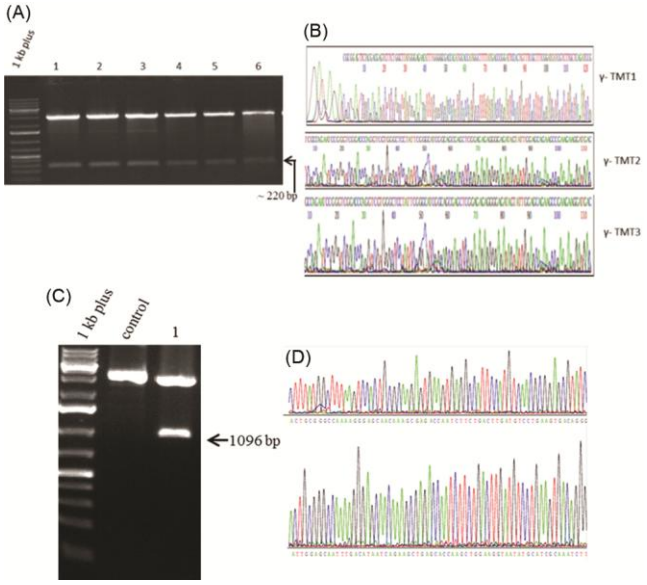

Fig. 1—Cloning and sequencing of γ-TMT sequences. (A & C) Restriction analysis of (A) γ-TMT partial CDS. (Lanes 1-3 and lanes 4-6 represent γ-TMT 1, γ-TMT 2 and γ-TMT 3 from Bragg and DS-2706 matured seeds, respectively); and (C) γ-TMT complete CDS. (uncut recombinant clone used as a control and lane 1 denotes release of γ-TMT complete CDS); and (B & D) Chromatogram illustrating the sequencing quality of (B) partial and (D) complete γ-TMT CDS, respectively]

and γ-TMT2 are located on the chromosome no. 12 at specific position *viz*., Glyma12g01680.1 and Glyma12g01690.1, respectively and these two genes are separated by a distance of 4 kb genomic sequence. However, γ-TMT3 gene is organized in chromosome no. 9 at specific position of Glyma09g35680.1. The differential nucleotide sequences of all the three *γ-TMT* genes of approximately 200 bp size encoding N-terminal domain were cloned and sequenced (Fig. 1 A & B). On account of sharing high similarity between the coding regions of *γ-TMT* genes, the complete coding region of *γ-TMT3* gene was cloned and sequenced (Fig. 1 C & D). The sequencing results showed 100% nucleotide similarity with the *γ-TMT* genes of *Glycine max* available in NCBI database (www.ncbi.nlm.nih.gov).

The deduced amino acid sequence of γ-TMT3 protein comprising 302 amino acid residues was selected for motifs and conserved domain analysis. MEME analysis revealed 3 conserved motifs when aligned with the γ-TMT protein sequences from selected plant species (Table 2), in which motif-I (20-113) and -II (116-245) were found to have SAM binding domain, whereas motif III did not correspond to any of the domains available in Pfam database (http://pfam.xfam.org) (Fig. 2). Based on the amino acid composition, it was

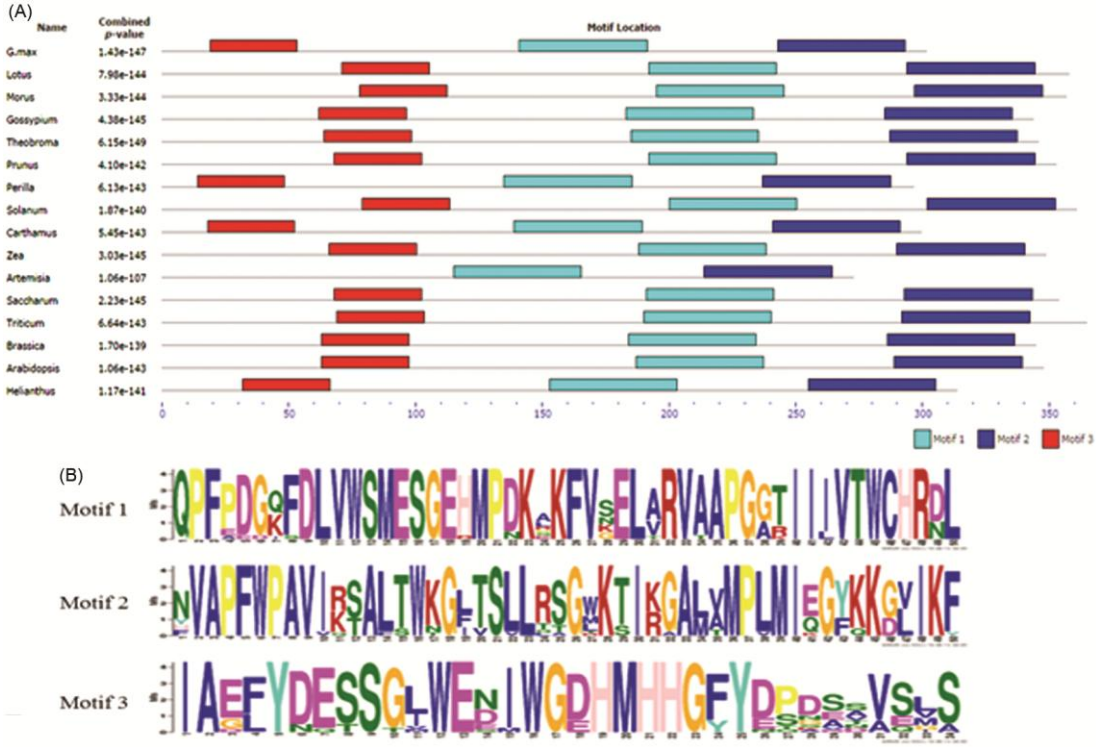

Fig. 2—Conserved motifs identification by MEME [(A) Amino acid sequence logos of motifs 1, 2 and 3, where size of residue is proportional to the conservation of the residue; (B) Distribution of conserved motifs in γ-TMT proteins. The name of each plant species and combined 'P' value are shown on the left side of the figure. Different motifs are indicated with different color boxes]

observed that *γ-TMT* gene of soybean was restricted to one phylogenetic group, which is a part of γ-TMTs from dicots (Fig. 3) and reported that these γ-TMTs are evolved from photosynthetic bacteria *Cyanobacterium stanieri*[34].

Expression analysis of *γ-TMT* genes during seed developmental stages of two contrast genotypes

Considering >90% homology between the three isoforms of *γ-TMT* genes and presence of SAM binding domain, it was anticipated that isoforms of γ-TMT might play crucial role in diverting the large amounts of γ-tocopherol to α-tocopherol in soybean seeds. To justify this, qPCR expression analysis of γ-TMT genes in all the three stages (30, 40 and 50 DAF) of seed development was carried out in Bragg and DS-2706. The results revealed that expression of γ-*TMT3* showed significant (P <0.05)

Difference between Bragg and DS-2706, as compared to *γ-TMT1* and *γ-TMT2* which did not differ significantly. The expression level of **γ-*TMT3*** in Bragg was 2-4.5 fold higher in all the developmental stages, as compared with DS-2706 (Fig. 4A). The *γ-TMT3* expression was 2, 3.4 and 4.5-fold higher, respectively at 30, 40 and 50 DAF in Bragg than in DS-2706 (Fig. 4A). Although fold change in the expression of *γ-TMT1* (<0.4) and *γ-TMT2* (<0.9) was higher in Bragg, as compared to DS-2706 (Fig. 4 B & C), the expression level was found to be non-significant (P <0.05). The qRT-PCR products of *γ-TMT* genes were also resolved through agarose gel to verify the desired size of the transcripts (Fig. 5). Earlier, in a study on *γ-TMT* isoforms expression in soybean leaves and seeds, the highest level of *γ-TMT3* expression was observed in fully matured seeds possessing high α-tocopherol content.

Our data suggested that *γ-TMT3* expression in Bragg might have coupled with accumulation of α-tocopherol in fully matured seeds. Difference in the NH2-domain of γ-TMT proteins (Fig. 1A) clearly showed that these NH2-domains represented the localization signals. For example, *in silico* analysis by ProtCompv. 9.0 (A tool which predicts the subcellular localization for plant proteins available in http://linux1.softberry.com) revealed the presence of chloroplast transit peptide from 1-23, 1-7 and 1-39 in γ-*TMT1*, *γ-TMT2* and *γ-TMT3*, respectively. However, Dwiyanti *et al*.[32] showed the presence of chloroplast peptide only in γ-*TMT1* by *in silico* analysis using Chloro P. Further studies are, however, needed to understand the heterogeneity in the expression of all three isoforms of γ-TMT in seeds and role of regulatory molecules in modulating their expression.

Correlation of γ-TMT gene expression with α-tocopherol accumulation

From our previous results, it was evident that *γ-TMT3* expression was significantly high in Bragg. In order to know the relationship between α-tocopherol

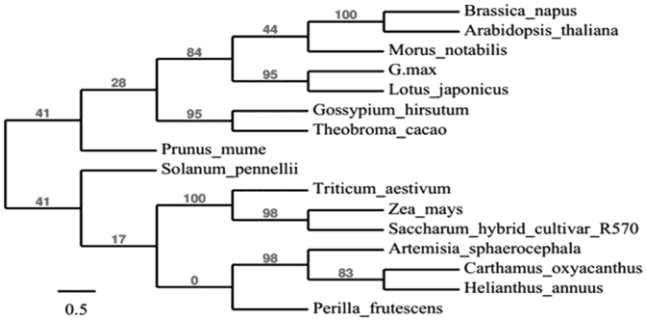

Fig. 3—Neighbor joining phylogenetic tree of γ-TMT proteins [Comparison of the deduced amino acid sequences of γ-TMT from soybean with γ-TMTs of plant species. An unrooted tree based on amino acid sequence similarity was obtained by using Mega 4.0 software. Bootstrapping was performed with 1,000 replicates and the bootstrap values (percent) were indicated above the supported branches]

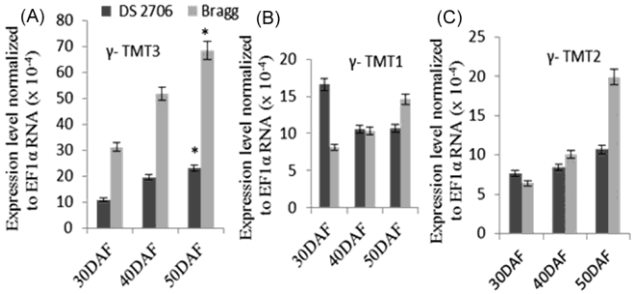

Fig. 4—Gene expression analysis of *γ-TMT3* (A), *γ-TMT1* (B) and *γ-TMT2* (C) during seed development [Expression level of γ-TMT transcripts were normalized with the values obtained for the internal control EF1α mRNA. Values represent the mean of three replicates ± SD. Asterisks show significant differences (P <0.05) between Bragg and DS_2706]

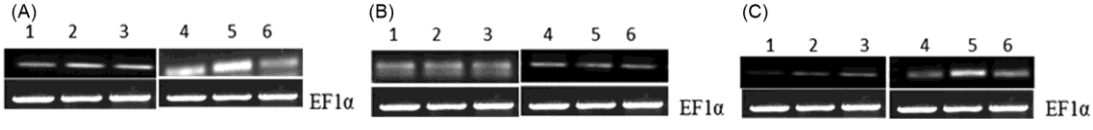

Fig. 5—The gel pictures showing qRT-PCR product of *γ-TMT3* [(A), *γ-TMT1* (B), *γ-TMT2* (C); Lanes 1-3 and lanes 1-6 denote Bragg and DS-2706, respectively at 30, 40 and 50 DAF]

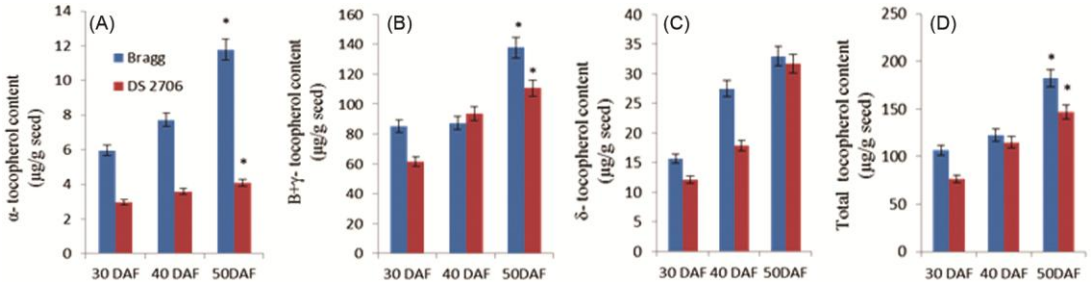

Fig. 6—Tocopherol concentration in developing seeds of Bragg and DS-2706 [Isoforms of tocopherol *viz*., (A) α-tocopherol; (B) β + γ tocopherol; (C) δ-tocopherol; and (D) total tocopherol were estimated in the seeds of 30, 40 and 50 DAF. [Data are represented as mean ± SD of the values obtained from triplicate experiments. For each seed developmental stage, significant differences (P <0.05) between the Bragg and DS-2706 are shown with asterisk]

accumulation and *γ-TMT3* expression, α-tocopherol as well as other forms of tocopherols (β + γ and δ-tocopherol) were estimated in the developing stages of seeds from Bragg and DS-2706, which were earlier subjected to gene expression studies. Significant difference (P<0.05) in the accumulation of α-tocopherol was observed between Bragg andDS-2706 at 50 DAF. Nearly 2, 4 and 7-fold increase in α-tocopherol content was observed, respectively at 30, 40 and 50 DAF in Bragg seeds than in DS-2706 (Fig. 6A). In addition, significant increase (P <0.05) in β + γ and total tocopherols was also observed in all developmental stages of seeds from Bragg, as compared to DS-2706 (Fig. 6 B & D).Though the increasing trend in δ-tocopherol accumulation was detected nearly 3 and 10 times at initial stages of seed development i.e. 30, 40 DAF, respectively in Bragg than in DS-2706. A difference in the accumulation of δ-tocopherol declined sharply between the two contrasting genotypes; only <1.5 times higher accumulation was observed in Bragg at 50 DAF than DS-2706 (Fig. 6C).

Our results thus clearly demonstrated that increase (11.78 µg/g) in α-tocopherol content at 50 DAF in Bragg was due to 6.5% increase in its concentration (concentration of α-tocopherol is calculated as ratio of α-tocopherol to that of total tocopherol), in comparison with DS-2706 (2.8%). Also, the data supported the relationship between *γ-TMT3* expression and α-tocopherol content in soybean seeds. Further work is, however, required to elucidate the functional differentiation of γ-TMT proteins and selectivity in their expression. Significantly higher β + γ tocopherol and total tocopherol content in Bragg at 50 DAF in our study was at variance with the observation of Dwiyanti *et al*.[32] who reported consistency in γ tocopherol and total tocopherol content between the selected genotypes. It was not surprising because genotype, environment and extraction method have been shown to strongly influence the tocopherol concentration in wheat[35], maize[36] and barley[37].

In conclusion, the study clearly demonstrated that out of the three *γ-TMT* genes, only the *γ-TMT3* showed significant expression in all the developmental stages of seed in Bragg genotype ('high' α-tocopherol) and the level of expression was positively correlated with α-tocopherol content.

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