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RESEARCH ARTICLE

Identification and characterization of genes involved in tocochromanol biosynthesis pathway through genomics and transcriptomics in durum and bread wheat

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Abstract

Lipid-soluble plant antioxidants, tocochromanol (tocols for short), are essential for the regulation of lipid peroxidation in chloroplasts and seeds. They are also referred to as Vitamin E. Wheat germ oil contains the highest percentage of tocols among these sources, but the information about the tocol biosynthesis gene is very minimal. Tocols, comprising tocotrienols and tocopherols, are beneficial for human health due to their antioxidant properties. The current research delved into the genome and transcriptome to identify genes involved in tocol biosynthesis in wheat. Ten different structural gene families were identified with a total of 78 isoforms, shedding light on the complex pathway of tocol synthesis. Genes showed distinct domain profiles, exon architectures, tissue localization, regulatory elements, chromosome location, synteny and phylogeny, indicative of their unique function. Differential expressions in 27 isoforms were identified through RNA sequencing of developing seedlings from high tocol (HI8663, HAU105) and low tocol content (NABIMG-9-Blue and PBW621) in durum and bread wheat varieties. This comprehensive approach not only provides insights into the natural diversity of tocol content in wheat but also paves the way for potential enhancements through breeding and genome editing techniques. Increasing tocol content in wheat could have significant implications for human nutrition and health.

Keywords: Tocochromanol, differential expression, lipid peroxidation, isoforms, wheat.

Introduction

Numerous organic compounds are produced by plants that not only play vital functions in plant cells but also are beneficial for nutrition purposes. One such class of compounds refers to a group of different derivatives, known as vitamin E. In plants, vitamin E refers to a group of lipid-soluble antioxidants that include tocotrienols and tocopherols. These compounds have a C20 isoprenoid-derived hydrocarbon tail attached to a polar chromanol head group derived from the shikimate pathway (Saffrané 2017). The primary distinction between tocopherols and tocotrienols is that tocopherols' hydrocarbon chain is entirely saturated, whereas tocotrienols have three trans double bonds. The amount and placement of methyl residues on the chromanol head group identify the four different forms of vitamin E found in plants (β, α, γ and δ). Three methyl groups are present in the chromanol ring of tocotrienols and tocopherols: one methyl group in the δ form, two methyl groups in the β and γ forms, but in different places. Tocochromanol is the collective term for four forms of tocopherols (α-T, β-T, γ-T, and δ-T) and four tocotrienols (α-T3, β-T3, γ-T3, and δ-T3) (Penna 2005). Thetocopherols

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form α-T is the most biologically active form of vitamin E and is primarily found in the human body. It has been presumed for a long time that tocochromanol production is unique to photosynthetic organisms, including algae, some cyanobacteria and plants. However, a recent study reported that malaria-causing, non-photosynthetic parasite *Plasmodium falciparum* also synthesizes α and γ tocopherols to avoid oxidative stress (Sussman et al. 2017). During storage, tocopherols increase the seed longevity by reducing the accumulation of lipid oxidation products (Sattler et al. 2004). In engineered tobacco leaves, tocotrienols help in reducing lipid peroxidation under low temperature and high light conditions (Matringe et al. 2008).

In tocol biosynthesis, two pathways, namely, shikimate and isoprenoidare implicated. The predecessor of shikimate is chorismate. In tocochromanol biosynthesis, the first step is the condensation of the polar aromatic head homogentisate (HGA) with different lipophilic polyprenyl pyrophosphates, which eventually establishes the type of tocochromanol. In the tocopherol biosynthesis pathway, condensation of HGA with phytyl diphosphate (PDP) takes place, while in the tocotrienol synthesis pathway condensation reaction is presumed to be with geranylgeranyl diphosphate (GGDP) instead of PDP. Geranylgeranyl is reduced from PDP with the help of geranylgeranyl reductase (GGR) either as the side chain of chlorophyll or as a diphosphate. In tocopherol pathway the first product is 2-methyl-6-phytylbenzoquinol (MPBQ) after prenylation of HGA with homogentisate phtyl transferase (HPT), while in case of tocotrienol it is 2-methyl-6-geranylgeranylbenzoquinol (MGBQ) after reaction of HGA with homogentisate geranylgeranyl transferase (HGGT) as shown in Fig. 1. Further methylation and cyclization occur to form the chromanol head group and second methylation (Hunter and Cahoon 2007).

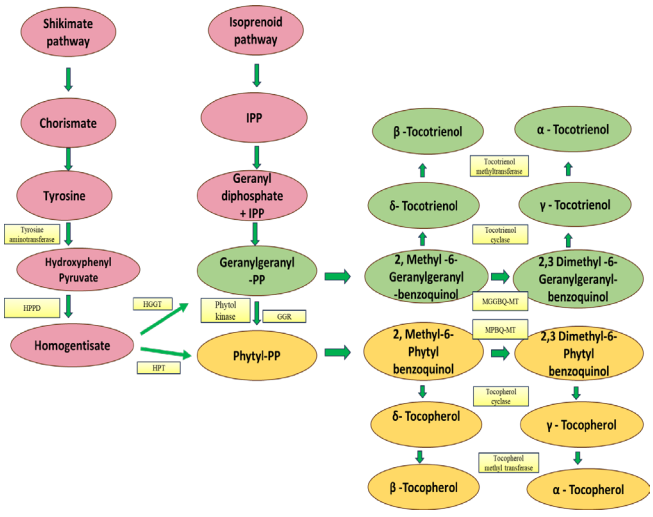

Fig. 1. Schematic representation of the tocopherol (vitamin E) biosynthesis pathway in wheat

A significant proportion of the world's population is suffering from mild to severe vitamin E deficiencies. During a survey conducted in poor and developed countries, 23% of the Seoul metropolitan population showed very low plasma α-T concentration (<12 µmol/L) that defines the threshold value for vitamin E deficiency. Vitamin E deficiency's effects on people's health haven't been thoroughly studied or reported yet. However, it is well-established that low plasma vitamin E is highly related to miscarriage during the first three months of a woman's pregnancy. Moreover, it has been shown that diets supplemented with vitamin E reduce miscarriage in pregnant women (Shamim et al. 2015). It also helps in the prevention of several diseases by suppressing cholesterogenesis, inhibiting lung cancer, reducing the risk of Alzheimer's disease and delaying brain aging (Fata 2014). Wheat (*Triticum aestivum*) is a staple food with wide use. Wheat germ oil is a source of vitamin E, containing both tocopherols and tocotrienols, with α-tocopherol being the most abundant. While the general vitamin E biosynthesis pathway is understood, research specifically focusing on the genes involved in this pathway in wheat is less extensive compared to other crops or model plants. This study aims to identify and characterize genes involved in tocol biosynthesis in seeds of durum (*Triticum durum* Desf.) and bread wheat (*Triticum aestivum* L.), focusing on their differential expression during development, ultimately seeking to identify key genes for enhancing tocol content.

Materials and Methods

Different wheat lines, including a variety, HI8663 of *Triticum durum* L. and three *T. aestivum* L. HAU105, NABIMG-9-Blue (Garg 2018) and PBW621 varieties were grown in the field of the National Agri-Food Biotechnology Institute (NABI), Mohali, India. Spikes from different developmental stages of seeds at 7, 14, 21 and 28 days after anthesis (DAA) were harvested, stored at -80°C and utilized for RNA isolation.

RNA isolation and RNA-Seq analysis

Sigma Spectrum plant's RNA kit was used to isolate RNA from three biological replicates, which were then processed through mRNA extraction, library preparation, and paired-end sequencing on an Illumina platform. After sequencing, Fast QC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 27 July 2024) was used to remove adaptor contamination and low-quality base pairs ($q < 20$) and BBDuk from the BBTools package (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 27 July 2024) was used to trim the reads from the 3' end. High-quality reads were mapped to annotated wheat transcripts retrieved from the ensemble plants (Ensemble version 47—April 2020) using the RNA-seq core analysis tool Salmon (PMCID: PMC5600148). Differential gene expression analysis was performed using tximport (Sonsen et al. 2015), which converts the transcript-level counts into gene-level

counts, and DESeq2 (Love et al. 2014) to analyse the data. For multiple hypothesis testing, the obtained p-*values* were corrected using the Benjamini and Hochberg technique implemented in the DESeq2 package. Genes having a false discovery rate (FDR) less than 0.05 were used in further analysis.

Genome-wide identification of structural genes

To determine the key structural genes involved in the wheat tocol biosynthesis pathway, the amino acid sequence of already known genes HPT, HGGT, HPPD, MPBQ, tocopherol cyclase (TC), gamma tocopherol methyl transferase (GTMT), phytol kinase (PK), GGDP, transaminases (TA) ands-adenosyl methionine (SAM)of *Arabidopsis thaliana* and *Oryza sativa* were retrieved. BlastP search was conducted against the wheat protein database obtained from the ensemble plant (https://plants.ensembl.org/Triticum_aestivum/Info/Index) using an e-value cutoff ≤ 0 and bit score > 100. Chromosome mapping was done on wheat chromosomes to analyse gene distribution. The graphical representation was drawn using the Map chart (version 2.2.0.0).

Characterization of the identified gene

Subcellular localisation, isoelectric points and conserved regions

The subcellular location of the major structural genes was predicted using WoLF PSORT (Horton et al. 2007) and Plant-mPLoc web servers (Chou & Shen, 2010). The molecular weights and isoelectric points were calculated using the isoelectric point calculator (IPC) (https://isoelectric.org/calculate.php, accessed on 5 October 2024). The conserved regions inside structural genes were located using the conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 6 October 2024).

Gene motifs, domains and cis-acting regulatory elements (CAREs)

For motif identification, the multiple expectation maximization for motif elicitation (MEME) programme (https://meme-suite.org/meme/ accessed on 15 October 2024) was used. To verify the presence of gene-specific domains, the found motif was queried against the SMART database. The motif identification parameters of width 6-50 and a maximum of 10 motifs were used. The cis-acting regulatory elements in the 1500 base pair upstream sequence (promoter region) were identified using the plantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 16 October 2024). CARE motifs were extracted, sorted and visualised for all available isoforms of the biosynthetic genes.

Phylogenetic relatedness and homology analysis

Protein sequences corresponding to differentially expressed genes (DEGs) were aligned using the MUSCLE alignment software (Edgar 2004) and a phylogenetic tree was constructed Neighbor-Joining (NJ) technique in MEGA X software (Kumar et al. 2018). In order to find orthologous genes to *T. aestivum*, the CDS sequences of close relatives (*T. turgidum* and *Aegilops tauschii*), which make the AB and D genomes, respectively, were accessed on 17 October 2024 from the Ensemble plant. To determine the synteny link, Circo's online server was used (http://mkweb.bcgsc.ca/tableviewer/visualize/, accessed on 27 July 2023).

qRT-PCR validation of tocol biosynthesis pathway genes during seed development

Genome-specific primers used in the tocol synthesis pathway were created using Primer3 (https://www.primer3plus.com/ accessed on 25 September 2024) (Supplementary Table S1: list of primers). Wheat alpha Actin (α-actin), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and alpha Tubulin (α-Tubulin) were used as an internal control. For the qRT-PCR, RNA was isolated from seeds collected at 7, 14, 21, and 28 DAA. cDNA was synthesized using a cDNA synthesis kit (Bio-Rad CFX96, USA). Diluted cDNA samples were subjected to quantitative real-time polymerase chain reaction (qPCR) using a 10 µl reaction mixture containing 0.5 µL cDNA and 9.5 µL of master-mix that includes SYBR green and forward-reverse primers. PCR was performed using a C1000 Touch thermal cycler (Bio-Rad, USA Amplifier). Amplification was set at different conditions, 95°C for 5 min, 95°C for 15 s, 60°C for 30 seconds, 72°C for 30 seconds and the fold change value based on $2^{-\Delta\Delta CT}$ values was used for the calculations using Actin as an internal control. GraphPad (version 5) was used for statistical significance at a 95% confidence level.

Results

In-silico identification of genes involved in tocolsbiosynthesis

A group of different structural genes from different enzyme super families catalyse the tocol biosynthesis pathway. These structural genes were retrieved from the Arabidopsis, barley, maize and riceand selected as query and the wheat proteome data was selected as database. BlastP search identified 14 TaMPBQ, 3TaTC, 3 Gamma-TaTMT, 3TaPK, 12 TaSAM, 21TaTA, 5 TaHPPD, 6TaGGDP, 3TaHPT, 3TaHGGT genes after filtering the result with 100-bit score and low e-value. The gene names, their respective family name and cellular localisation characterised through WoLF PSORT and Plant-mPLoc web servers are given in Table 1. The differentially expressed genes in high tocol (HI8663, HAU105) vs low tocol lines (NABIMG-9-Blue, PBW621) were subjected to the additional study. The genome and chromosomal distribution of genes found through the ensemble plant and drawn by the Map chart are shown in Figure 2. Maximum no. of genes was localised on the sub genome B(11)

followed by subgenome A (10) and least in sub-genome D (6) (Fig. 2). All the structural genes of this pathway were mapped on 21 chromosomes (Fig. 2). High gene density was observed on homoeologous group-6 chromosomes with 6A and 6B having four and 6D three. Low density was found on homoeologous group-4 chromosomes. Chromosomes 1, 3 and 7 had equal distribution of genes (3 each). In case of homoeologous group-1 chromosomes, one gene was present on 1A and two genes on 1B, while homoeologous group-3 had equally distributed genes on A, B, and D subgenomes (1 each). Chromosome no. 7 had 2 genes on the A subgenome and one gene was present on the B subgenome. In case of homoeologous group-5, there were two, three genes and one gene present on 5A, B and D, respectively. Among all the gene families, TaTA was maximum at subgenome A (2). TaSAM was equally present on subgenomes A and B (2 each). The TaHPPD gene family showed equal distribution on A, B and D sub-genomes. TaMPBQ was equally present on subgenomes B and D (3 each) and A had 2 genes. TaHGGT and TaGTMT genes were absent on subgenome B and D, while TaHPT and TaTC genes were absent on subgenome A and D. In the case of the TaSAM family, no genes were present on subgenome D.

Physiological properties and domain characterization

The molecular weight, domain organisation, isoelectric point, and subcellular localization of genes identified through WoLF PSORT and Plant-mPLoc are shown in Table 1. Domain analysis using the CDD tool identified that HPT genes belonged to the UbiA superfamily, HGGT to PT_UbiA, HPPD to HppD, and MPBQ to SmtA. Further, TC belonged to the Tocopherol_cycl superfamily, GTMT to PLN02244, PK to CdsA, GGDP reductase to GG-red-SF, TA totyr_amTase_E and SAM to AdoMet_MTase. The highest isoelectric point was observed in PK and the molecular weight in TC. Most of the genes were chloroplast-specific, while a few were localised in mitochondria, plastid and cytoplasm.

Differential expression analysis

Differentially expression of high tocol wheats (HI8663 and HAU105) *vs* low tocol (PBW621 and NABIMG-9-Blue) was analysed using DESeq2 was used to prepare a heat map (FDR<0.05) as shown in Figure 3. Among the three TaTC genes, only one gene showed differential expression. Out of 14 TaGTMT genes, only one gene (TaGTMT1) in the Gamma-TMT gene family was differentially expressed. Out of 14 MPBQ genes, four genes (TaMPBQ3, TaMPBQ5, TaMPBQ9, TaMPBQ12) were highly expressed in high tocol wheat varieties, while four genes (TaMPBQ1, TaMPBQ10, TaMPBQ13, TaMPBQ14) were highly expressed in low tocol content varieties. In the case of 12 putative SAM genes, four genes (TaSAM1, TaSAM3, TaSAM5, TaSAM6) were differentially expressed. Out of 21 putative TA genes, three genes (TaTA1, TaTA14, TaTA16) were identified as differentially expressed

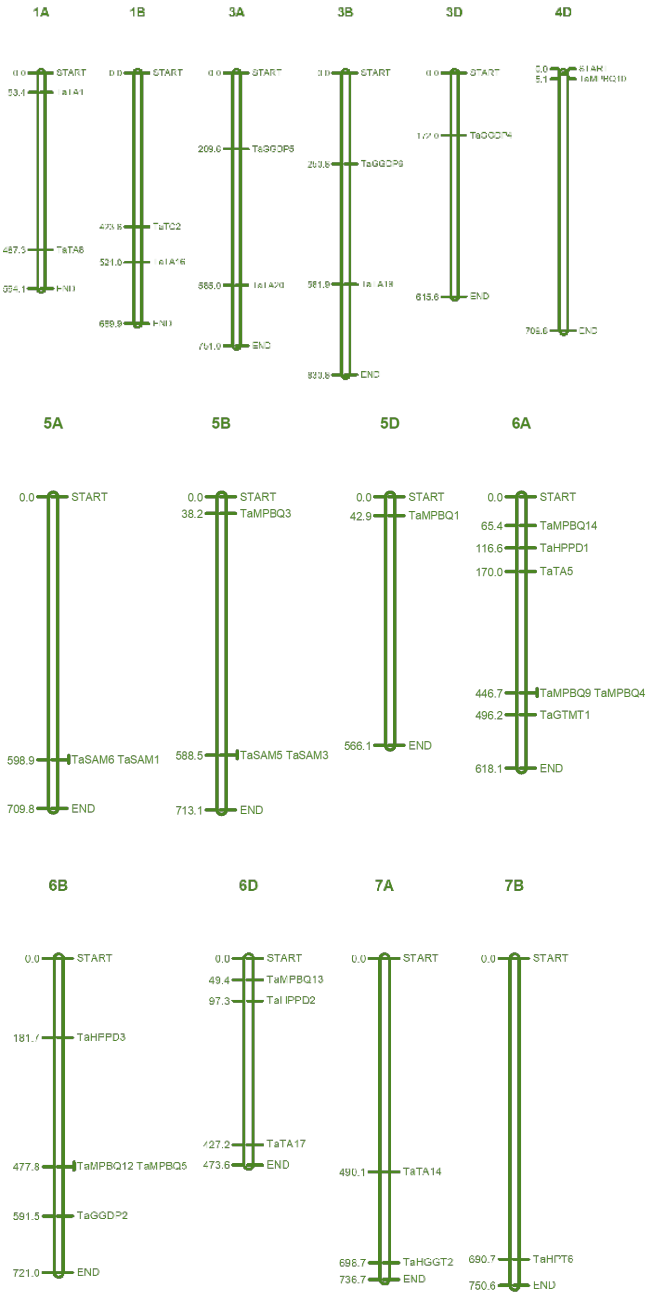

Fig. 2. Chromosomal localization of tocol biosynthesis DEG isoforms of the wheat genome

in high tocol wheat varietieswhile one gene (TaTA17) was highly expressed in low tocol wheat varieties. Among the six genes of HPPD, three were differentially expressed. Out of 5 HGGT genes, one gene (TaHGGT2) was differentially expressed in high tocol wheat varieties. Out of six HPT genes, one gene (HPT6) was differentially expressed. The expression pattern of putative DEG isoforms was inconsistent across the four wheat lines. Unexpectedly few isoforms of tocol biosynthesis showed higher expression in low tocol wheat varieties. Each biosynthetic gene's expression pattern of potential DEGs was confirmed by quantitative real-time

Table 1. Physiological properties, subcellular localization and detailed information of the structural genes of the tocol biosynthesis pathway in wheat

S.No	Gene	Abbreviation	Isoelectric point (Min-Max)	Predicted Molecular Mass KD (Min–Max)	Site	Superfamily
1	Homogentisate phytyltransferase	HPT	9.59-10.04	41.59-43.99	Plastid	UbiA superfamily
2	Homogentisate geranylgeranyltransferase	HGGT	8.06-9.44	29.80-50.68	Plastid	PT_UbiA superfamily
3	4-Hydroxyphenylpyruvate dioxygenase	HPPD	5.32-6.28	43.57-50.04	Chloroplast	HppD superfamily
4	2-Methyl-6-phytyl-1,4-hydroquinone methyltransferase	MPBQ	8.88-8.91	38.77-39.08	Mitochondria	SmtA superfamily
5	Tocopherol cyclase	TC	5.68	1767.41	Chloroplast	Tocopherol_cycl superfamily
6	Gamma-tocopherol methyl transferase	Gamma TMT	6.2-7.002	39.48-40.95	Chloroplast	PLN02244 superfamily
7	Phytol kinase	PK	4.72-10.41	21.36-33.59	Chloroplast	CdsA superfamily
8	Geranylgeranyl diphosphate reductase	GGDP reductase	5.8-9.04	36.17-50.62	Chloroplast	GG-red-SF superfamily
9	Tyrosine transaminase	TA	5.18	47.616	cytoplasm	tyr_amTase_E superfamily
10	S-adenosyl-L-methionine	SAM	5.21	47.475	Cytoplasm	AdoMet_MTases superfamily

polymerase chain reaction (qRT-PCR; Figure 4). DEGs of individual biosynthetic genes have been found to exhibit a similar pattern to that of transcriptome data. According to qRT-PCR data, high tocol wheat lines had higher expression of TaHPT6, TaSAM3, TaHGGT2, and TaTA5, while low tocol wheat lines had higher expression of TaHPPD2 and TaSAM3. To elucidate the contribution of the D subgenome to tocol biosynthesis, in the durum wheat variety, HI8663 (genomic constitution AABB) was used as a control. When comparing low-tocol hexaploid wheat (genomic constitution AABBDD) varieties with HI8663, it was observed that most of the genes upregulated in HI8663 were distributed across the A and B subgenomes. The expression level of DEGs identified on the D sub-genome of hexaploid wheat remained low in both high tocol wheat (HAU105 and HI8663), suggesting limited transcriptional activity of D-genome-derived genes associated with tocol biosynthesis under the tested conditions. Differential gene expression analysis revealed that few DEGs localized at A and B subgenomes had lower expression in high tocol wheat as compared to low tocol wheat.

Cis-acting regulatory elements (CAREs)

The promoter region of the identified genes was investigated for CAREs using the Plant CARE database.CARES were found to be involved in core promoter function, enhancer function, as well as tissue-specific and trait-specific functions. The upstream region of the TaSAM5 and TaTA16 gene showed a higher no. of CAREs compared to other biosynthetic genes. Root-specific CAREs were found only in the TaGGDP6 and TaGTMT1 gene families. Wound-responsive CARE element is absent on all gene families except TaHPPD3. Most of the CAREs were stress responses such as drought, anoxic, light, low temperature, salicylic acid, abscisic acid etc. (Supplementary Fig. 1). The CAREs of few gene families were involved in endosperm expression (TaTA16, TaGGDP5, TaSAM3, TaMPBQ5, TaMPBQ9, TaMPBQ12) whilea few others showed seed specific regulation (TaSAM5, TaMPBQ9 and TaGGDP2).

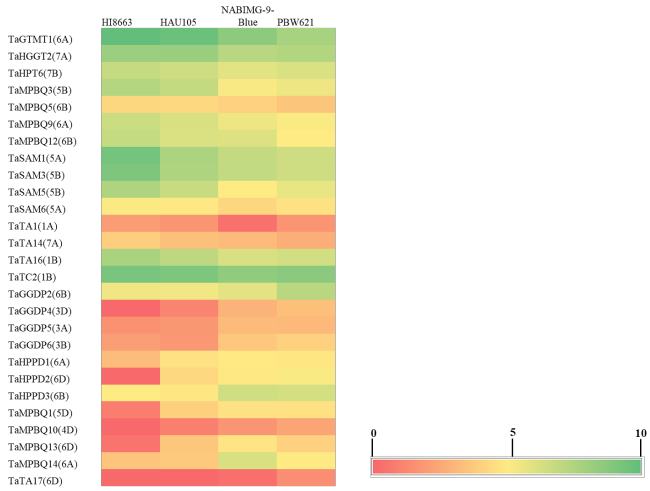

Fig. 3. Heatmap showing the relative expression levels of key tocol biosynthesis genes across different colored and non-colored wheat lines

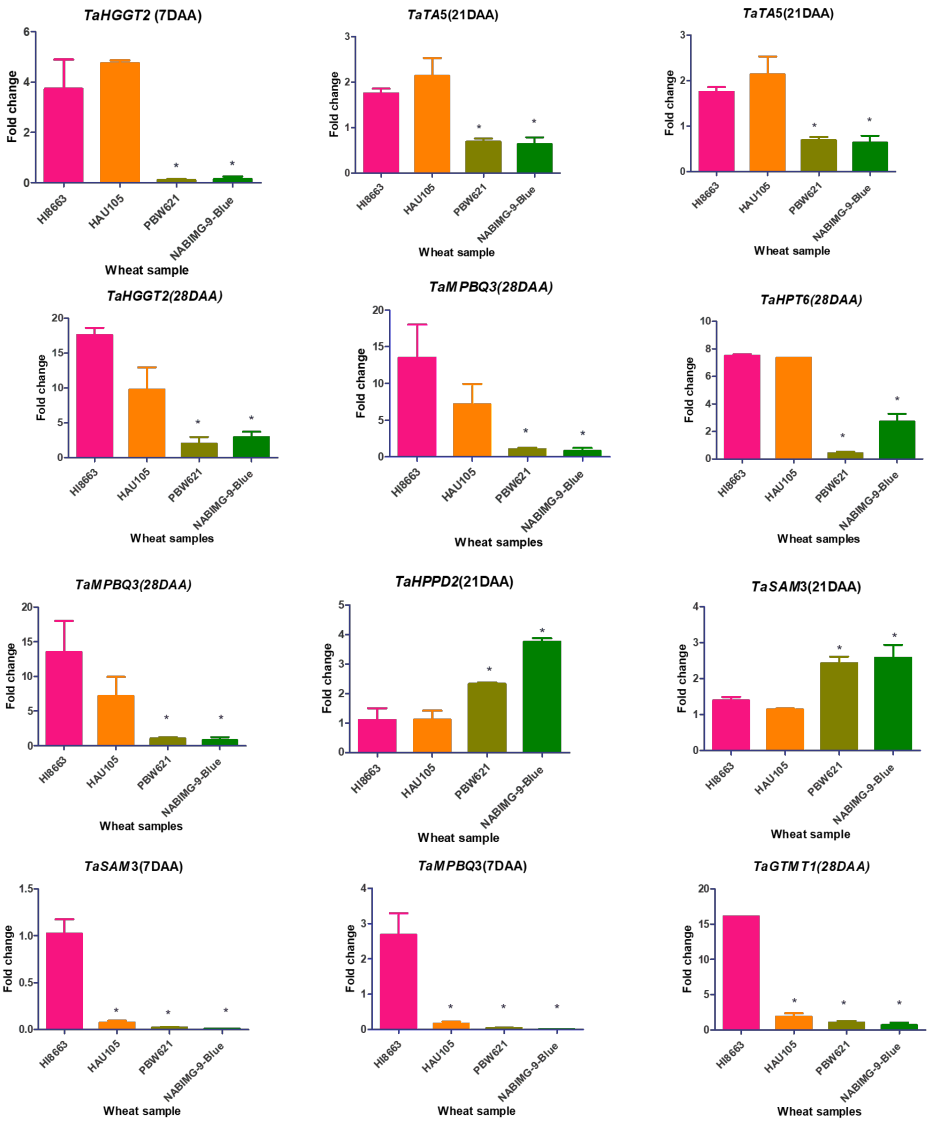

Fig. 4. Expression profiling of tocol biosynthesis genes in wheat using quantitative real-time PCR (qRT-PCR). Significant values are depicted by *, depicting the p-value <0 .05. At 28 DAA, genes such as TaHPT6, TaTA5, TaSAM3, TaHGGT2, TaMPBQ3; at 21 DAA, TaTA5; and at 7DAA, TaHGGT2 showed upregulation in high-tocol lines, whereas TaHPPD2 and TaSAM3 (21DAA) were upregulated in low-tocol lines at 21DAA. Appearance of a third group of patterns revealed high expression in one of the two high-tocols line only, which may be due to genotype specificity. Overall, in high-tocopherol lines, enzymes involved in the initial steps are necessary during the early stages (7–14 days), while late-stage enzymes, such as methyltransferases, are required toward the end. Early synthesis of these late-stage enzymes could lead to degradation before they are actually needed.

Gene Structure and Phylogenetic Analysis

Gene structure and motifs of DEGs determined using MEME Suite tools are shown in Figure 5(A). During analysis of gene structure of all gene families, the TaTC2 gene has the largest size (>10Kb) and has equal number of introns and CDS.Out of four GGDP DEGs, three DEGs were intron-less (TaGGDP5, TaGGDP6 and TaGGDP4) and one DEG (TaGGDP2) has two introns and 3 CDS in its motifs. A similar pattern to TaGGDP2 was observed in the case of TaMPBQ3. In case of TA DEGs, approximately 4 to 6 introns and 5-7 CDS regions were present. The number of introns and CDS was equal in the case of the TaMPBQ5 gene (3 each). The maximum number of CDS regions was present in TaHPT6, while the maximum introns were present in TaTC2. The same number of CDS regions (6) and introns (5) were found in the case of TaTA1, TaMPBQ14, TaMPBQ9, TaGTMT1, TaMPBQ12 and TaMPBQ13. DEGs of TaSAM5 and TaHGGT2 showed a similar size, pattern and number of introns and CDS regions. The smallest size motifs were observed in the case of TaHPT6 (< 2kb). In case of MPBQ DEGs family genes, all DEGs have approx. 3-6 CDS regions and 2-5 introns. Few gene DEGs (TaGGDP5, TaGGDP6 and TaGGDP4) were intron-less. The UTR region was absent in case of TaGGDP5 and TaHPT6, respectively.

The 27 DEGs of *T. aestivum* were investigated for phylogenetic relation with other species (*Oryza sativa, Zea*

mays, Hordeum vulgare, Triticum turgidum and Arabidopsis thaliana). A phylogenetic tree of each target gene was constructed using the muscle algorithm by using the multiple sequence alignment is presented in Figure 5(B). Genes with similar functions or expression patterns were clustered together in different groups (1-8). Phylogenetic analysis demonstrated that most genes clustered according to their respective groups, indicating conserved evolutionary relationships; however, the presence of intermixed domains among different groups suggests possible domain rearrangements or evolutionary convergence. The putative SAM genes were clustered in groups 1 and 7, TC and gamma TMT genes were clustered in group 4, HPT and HGGT genes were clustered in group 3, respectively. Groups 5 and 6 consist of TA genes and HPPD genes. The putative GGDP genes were clustered in group eight. The phylogeny analysis indicated that tocol biosynthesis genes evolved independently from each other and these all may have different functional roles in different pathways.

Since wheat evolved from hybridization, we further tried to understand the synteny relationship between wheat and its genomic contributors. Further chromosome-wise classification of orthologous tocol biosynthesis pathway (*Ae. tauchii* and *T. turgidum*) was done by circular ideogram of Circos (Fig. 5C). The A subgenome (chromosomes 1A, 3A, 4A) and B subgenome (1B, 3B, 4B) exhibit distinct yet complementary patterns in housing genes involved in the tocopherol biosynthesis pathway. Circos ribbons connecting 1A and 1B to SAM, TA, and MPBQ genes show a high degree of interconnectivity, implying potential co-expression or coordinated regulation of these genes across the A and B subgenomes. Notably, 1B acts as a key hub, with dense linkages to SAM1–SAM6, indicating its major role in modulating methyl group transfers, which are critical for α-tocopherol production. In contrast, 1A contributes multiple TA genes (TA1, TA14, TA16), possibly providing precursors or acting as upstream regulators in the pathway. The A genome seems more involved in precursor generation and intermediate processing, while the B genome contributes significantly to the maturation and functional diversification of tocol compounds.

Discussion

The study of tocols has significantly surged in recent decades due to increased awareness of their health implications in relation to specific food items and dietary patterns. Although vegetable oils are the primary source of tocols, most cereal grains, such as barley, oat, wheat, rye, and rice, have been found to contain significant levels of these compounds (Fratianni et al. 2013). In wheat, wheat germ oil is a special oil with its strong nutritional profile and vitamin E enrichment (Naila Siraj, 2021). Various studies have been done on wheat germ oil for its policosanol (Irmak et al. 2006), riboflavin, glutathione, thiamine, sterols, flavonoids, octacosanols,

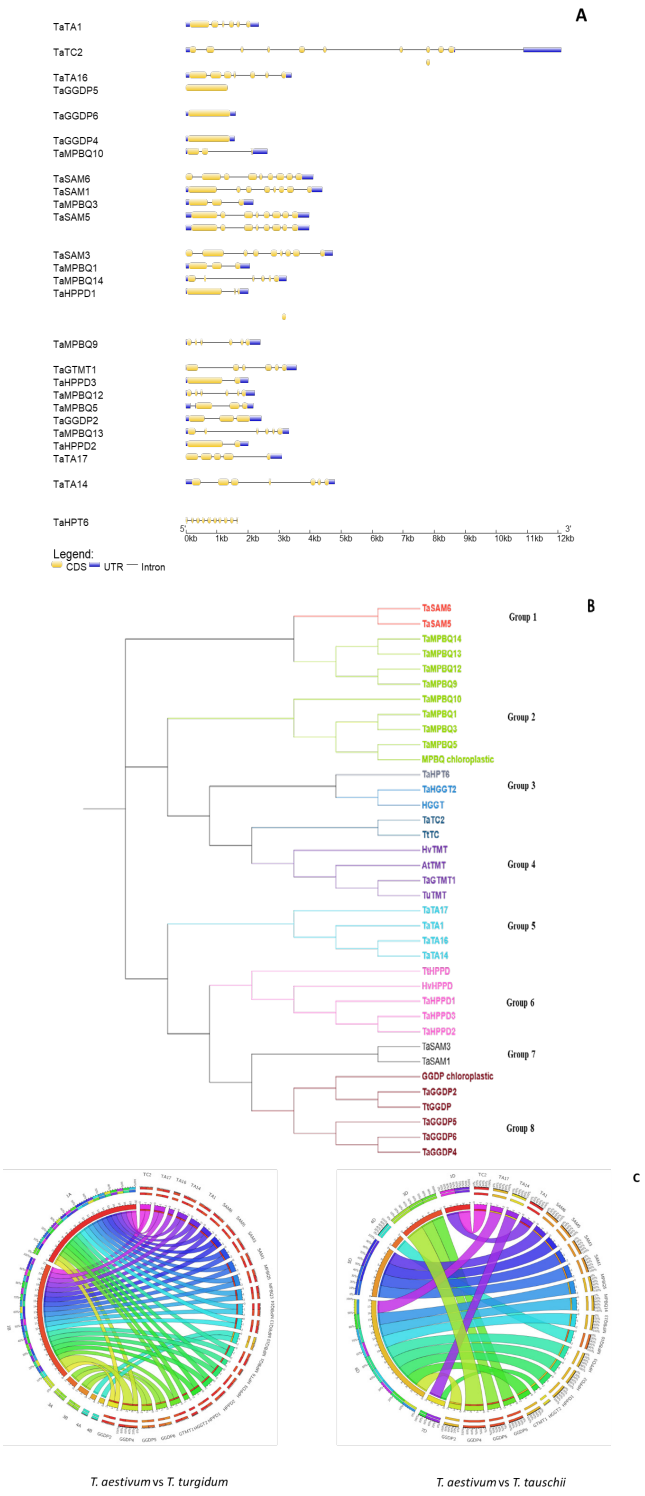

Fig. 5. Structural and comparative analysis of tocol biosynthesis genes in wheat. (A) Exon–intron structure of target genes visualized using GSDS; yellow boxes indicate exons; blue lines indicate introns. (B) Phylogenetic clustering of wheat and related cereal tocol biosynthesis genes based on sequence homology; different colors represent distinct gene groups. (C) Synteny analysis showing genomic relationships of T. aestivum genes with their orthologs in T. tauschii and T. turgidum; chromosomes represent ancestral genomes; gene names represent wheat genome locations

steryl ferulates (Kumar and Krishna 2013) and numerous enzymes (Zhu et al. 2011). Numerous investigations were carried out to find genes linked to tocol biosynthesis, such as transcriptome analyses in barley, rice, oats, and oilseed species, genotyping-by-sequencing (GBS) for a wild barley panel, and genome-wide association study (GWAS) employing single nucleotide polymorphisms (SNPs) obtained from Illumina arrays for a cultivated barley mini-core panel (Mahalingam et al. 2020; Song et al. 2024).

Due to a lack of information, very few studies are available on wheat for its tocopherol content and about the genes responsible for its biosynthesis pathway, while, vitamin E biosynthesis pathway has been thoroughly explored in model species (DellaPenna et al. 2005; Collakova et al. 2003; Quadrana et al. 2013). Enhancing γ-tocopherol in oil crops is crucial for preventing oil peroxidation and hence improving oil quality (Abbadi et al. 2004; Goffman et al. 2007). Therefore, enhancing the tocopherol content in crop plants is a critical objective due to its nutritional value for cattle and humans (Fritsche et al. 2017).

Role of different gene families in tocol accumulation in wheat

Tocol accumulation in wheat is influenced by multiple gene families that regulate different aspects of its biosynthesis, transport, and storage. Through the use of genetic engineering (CRISPR/Cas9, molecular breeding), it has been possible to enrich the alpha tocopherol content of Arabidopsis, maize, soybean, sorghum, rice, barley, tomato, tobacco, lettuce, potato, and canola by overexpressing a single gene or multiple genes together (co-expression). HGGT/HPT (overexpressed/CRISPR-Cas9), TC (overexpressed), and HPPD (overexpressed) were the main genes implicated (Supplementary Table 1).

In our most recent work, we used an *in-silico* study to compare the expression levels of the structural genes involved in tocol production across several wheat varieties. During our research, we found 10 different structural gene families (HPT, HGGT, HPPD, MPBQ, TC, GTMT, PK, GGDPR, TA, SAM) are involved in tocol biosynthesis in wheat (Konda et al. 2020; Karunanandaa et al. 2005; Motohashi et al. 2003). The maximum number of genes was identified in the case of MPBQ, followed by the TA gene family. In this study, the heatmap revealed that maximum MPBQ genes are highly expressed in high-tocopherol wheat varieties (HI8663 and HAU105). Few MPBQ genes, which are low expressed in high tocol varieties, are present on subgenome D and their value is close to zero. These findings suggested a potential role of MPBQ in tocol accumulation (Wu et al. 2022; Park et al. 2019). The qRT-PCR result further confirmed the high expression of this gene in high-tocopherol wheat as compared to low, supporting this hypothesis. Further, HGGT and HPT were shown to have the same substrate specificities (homogentisate), leading to the formation of tocotrienols and tocopherols, respectively (Yang et al. 2010). A previous study reported that mutant HPT and HGGT genes in Hordeum vulgare result in a reduction of tocopherol and tocotrienol content (Zeng et al. 2020). Some other studies also reported that high HGGT expression helps in the accumulation of higher tocotrienol content in rice (Yang et al.2011; Yuan et al.2020; Huang et al. 2013). This demonstrated that HGGT is, in fact, the primary enzyme responsible for grain's T3 production (Matsuzuka et al. 2013). In this study, we observed that both the TaHGGT2 and TaHPT genes were highly expressed in wheat lines with elevated tocol content, which suggests their potential roles in tocol biosynthesis. The results from qRT-PCR analysis confirmed the high expression levels of both genes in the high tocol wheat lines, supporting the observed correlation between gene expression and tocol content. These findings suggest that TaHGGT2 could serve as a promising candidate for future studies aimed at enhancing tocol concentrations in wheat. Further research could focus on overexpressing TaHGGT2 in wheat to assess its potential in increasing tocol content. Additionally, exploring the regulatory mechanisms that control the expression of these genes could offer deeper insights into the biosynthesis pathway, ultimately providing a more comprehensive understanding of how to enhance tocol production in wheat.

The p-hydroxyphenyl pyruvate (HPP) is converted to homogentisic acid (HGA) by the HPPD, a critical enzyme in the tocopherol biosynthesis pathway. HGA serves as a precursor for the tocol molecules. The study by Jiang et al. (2017) demonstrated that overexpression of the HPPD gene, specifically the MsHPPD gene from Medicago sativa, resulted in significantly elevated levels of β-tocotrienol and overall vitamin E in alfalfa leaves. In the context of wheat, our qRT-PCR analysis of TaHPPD1 expression in various wheat lines revealed differential expression levels, with higher expression observed in the low-tocol wheat lines PBW621 and NABIMG-9, and lower expression in the high-tocol wheat lines HI8663 and HAU105. These findings suggest that the regulation of HPPD expression may play a significant role in determining tocopherol content in wheat. Moreover, the chloroplast-specific localization of this gene further highlights its potential importance in photosynthesis-related processes and overall plant metabolism, which could influence tocopherol production under varying environmental conditions. The contrasting results in HPPD expression and tocopherol content across different wheat lines are likely due to a combination of environmental, genetic and biochemical factors. Further research into the molecular regulation of HPPD expression and its interaction with other metabolic pathways in wheat could provide more insight into these differences (Sanchez et al. 2024).

Aromatic aminotransferases (Aro ATs) are PLP-dependent

enzymes that catalyse the transamination of aromatic amino acids and keto acids, contributing to the biosynthesis of tocopherols and other plant secondary metabolites such as flavonoids, lignin, and auxin (Riewe et al. 2012). In our study, high-tocol wheat lines showed significantly elevated Aro AT expression, particularly on subgenome A, indicating subgenome-specific regulation, a well-established feature in polyploid wheat (Chao et al. 2014; Huang et al. 2016). This aligns with prior findings that AAA-derived pathways are involved in tocopherol biosynthesis (Koper et al. 2023; Wang et al. 2021). Additionally, we examined GGDP reductase genes, which synthesize phytyl-diphosphate (PDP)—a crucial precursor for tocopherol production. These genes targeted to chloroplasts showed an intron-less structure that may support efficient transcription (Upadhyaya et al. 2021). This supports earlier findings that GGDP reductase could act as a regulatory step in tocopherol biosynthesis (Ajjawi and Shintani 2004), with resource allocation potentially influencing chlorophyll *versus* tocopherol synthesis (Qin et al. 2024). Moreover, the gamma tocopherol methyl transferase (GTMT) gene, especially TaGTMT1, showed strong expression in high-tocol lines, correlating with α-tocopherol enrichment. Previous overexpression studies in tobacco and soybean confirmed that GTMT enhances α-tocopherol levels and improves resistance to salt and heavy metal stress (Dwiyanti et al. 2011). Our results collectively highlight the potential of TaGTMT1, Aro ATs, and GGDP reductase genes as key targets for engineering wheat cultivars with elevated vitamin E content and improved resilience to environmental stress. In the present study, DEG from the tocopherol cyclase (TC) family, TaTC2, was identified, which exhibited higher expression in high tocol wheat lines. The mutation of TC has been linked to tocopherol deficiency and the accumulation of intermediate compounds such as 2,3-di, methyl-6-phytyl-1,4-benzoquinone (DMPBQ), as demonstrated by Kanwischer et al. (2005).

The domain analysis of TaTC2 identified it as part of the "tocopherol_cycl superfamily," suggesting its role in tocopherol biosynthesis, specifically in forming the tocopherol chromanol ring. The present study identified several cis-regulatory elements, such as CARE elements, that regulate endosperm expression and are involved in stress responses, drought and salinity, as well as ABA signalling. Previous research on rice (OsVTE1) confirmed that TC expression is induced by abiotic stress and plant hormones like salicylic acid and ABA (Ouyang et al. 2011). Beyond antioxidant functions, tocopherols play roles in cellular signalling, glucose metabolism, and stress responses (Rey et al. 2021). The increased expression of TaTC2 in high-tocol wheat lines likely contributes to the elevated tocopherol content. However, further research is needed to fully understand the regulatory mechanisms behind TaTC2 expression and its role in tocopherol isoform accumulation. These insights could enhance tocopherol content in wheat, improving its nutritional value.

The circular chart illustrates the diversity of genes involved in tocol biosynthesis in wheat. Key gene families include HPPD, MPBQ, GGDP, GTMT, SAM, and others. The MPBQ gene family is notably abundant, suggesting its central role in the pathway (Falk and Munné-Bosch 2010). Multiple entries of GGDP and HPPD genes reflect their importance in precursor synthesis (Collakova and DellaPenna 2001). The GTMT family, crucial for α-tocopherol production, also shows significant representation (Shintani and DellaPenna 1998). Presence of genes from other species like AtTMT and HvTMT aids in comparative analysis (Cheng et al. 2003). SAM genes, involved in methyl group transfer, are also well represented (Gilliland et al. 2006). This diversity highlights the genetic complexity of tocopherol biosynthesis. It offers promising targets for improving vitamin E content through breeding. Such insights are vital for enhancing wheat's nutritional and stress-resilience traits.

Supplementary material

Supplementary Table S1 and Supplementary Figure 1 are provided which can be accessed at www.isgpb.org

Authors' contribution

Conceptualization of research (MY, MG); Designing of the experiments (MY, AS, MG); Contribution of experimental materials (MY, AS); Execution of field/lab experiments and data collection (EC, MY); Analysis of data and interpretation (AS, AT, BS, MY); Preparation of the manuscript (MY, RN)

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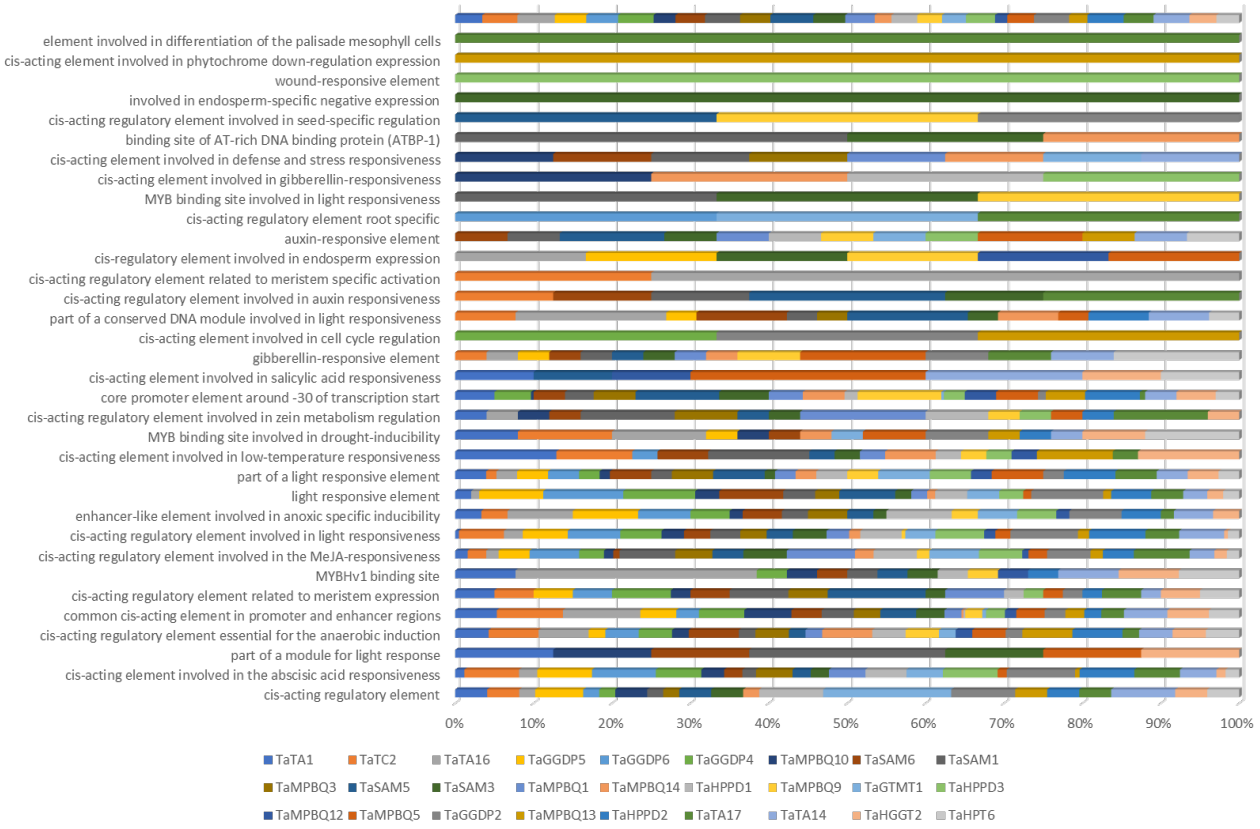

Supplementary Fig. 1. Most commonly occurring cis-regulatory elements in target genes

Supplementary Table S1: Genes involved in tocol enhancement across crops

Gene/Model species used for enhancing tocol content	Enhancement approach	References	Total genes identified in wheat	Genes showed higher expression in high tocol content wheat varieties
1. Homogentisate geranylgeranyl transferase (Maize and Arabidopsis)	Molecular breeding	Cahoon et al., 2003	5 (*TaHGGT*1-5)	*TaHGGT2*
1. 4-Hydroxyphenylpyruvate Dioxygenase (Arabidopsis)	Overexpression	Jiang et al., 2017	5 (*TaHPPD*1-5)	-
2. *OsHPPD* (*Oryzae sativa*)	CRISPR/Cas9	Li et al., 2018		
1. Homogentisate Phytyltransferase (Arabidopsis and Tobacco)	Overexpression	Collakova & DellaPenna, 2001	6 (*TaHPT*1-6)	*TaHPT6*
2. *OsHPT* (*Oryzae sativa*)	CRISPR/Cas9	Li et al., 2018		
1. γ-tocopherol methyltransferase (Soybean)	CRISPR/Cas9	Zhang et al., 2021	3 (*TaGTMT*1-3)	*TaGTMT1*
2. γ-TMT (Maize)	Molecular breeding	Diepenbrock et al., 2017		
1. Methyl phytyl benzoquinone methyl transferase (Maize)	Natural allelic variation via GWAS	Diepenbrock et al., 2017	14 (*TaMPBQ*1-14)	*TaMPBQ3*, *TaMPBQ5*, *TaMPBQ9*, *TaMPBQ12*
1. Tocopherol cyclase (Arabidopsis)	Overexpression	Shintani & DellaPenna, 1998	3 (*TaTC*1-3)	*TaTC2*
2. TC (*Oryzae sativa*)	Overexpression	Cho et al., 2005		
1. S-adenosyl methionine (Arabidopsis, wheat)	Expression analysis	Alessi et al., 2018	12 (*TaSAM*1-12)	*TaSAM1*, *TaSAM3*, *TaSAM5*, *TaSAM6*
1. Aromatic Aminotransferases (Arabidopsis, wheat)	Expression correlation with tocopherols	Maeda & Dudareva, 2012	17 (*TaTA*1-17)	*TaTA1*, *TaTA14*, *TaTA16*