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Production of tocotrienols in seeds of cotton (*Gossypium hirsutum* L.) enhances oxidative stability and offers nutraceutical potential

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Abstract

Upland cotton (*Gossypium hirsutum* L.) is an economically important multi-purpose crop cultivated globally for fibre, seed oil and protein. Cottonseed oil also is naturally rich in vitamin E components (collectively known as tocochromanols), with α- and γ-tocopherols comprising nearly all of the vitamin E components. By contrast, cottonseeds have little or no tocotrienols, tocochromanols with a wide range of health benefits. Here, we generated transgenic cotton lines expressing the barley (*Hordeum vulgare*) homogentisate geranylgeranyl transferase coding sequence under the control of the *Brassica napus* seed-specific promoter, napin. Transgenic cottonseeds had ~twofold to threefold increases in the accumulation of total vitamin E (tocopherols + tocotrienols), with more than 60% γ-tocotrienol. Matrix assisted laser desorption ionization-mass spectrometry imaging showed that γ-tocotrienol was localized throughout the transgenic embryos. In contrast, the native tocopherols were distributed unequally in both transgenic and non-transgenic embryos. α- Tocopherol was restricted mostly to cotyledon tissues and γ-tocopherol was more enriched in the embryonic axis tissues. Production of tocotrienols in cotton embryos had no negative impact on plant performance or yield of other important seed constituents including fibre, oil and protein. Advanced generations of two transgenic events were field grown, and extracts of transgenic seeds showed increased antioxidant activity relative to extracts from non-transgenic seeds. Furthermore, refined cottonseed oil from the two transgenic events showed 30% improvement in oxidative stability relative to the non-transgenic cottonseed oil. Taken together, these materials may provide new opportunities for cottonseed co-products with enhanced vitamin E profile for improved shelf life and nutrition.

Introduction

Four species of *Gossypium* L. are cultivated in the tropical and subtropical regions of the world because of their great economic value. Two are diploid ($2n = 2x = 26$) 'old world' species (*G. arboreum* L. and *G. herbaceum* L.) that are cultivated in some local regions in Asia and Africa. The other two are polyploid ($2n = 4x = 52$) 'new world' species (*G. barbadense* L. and *G. hirsutum* L.) that today are cultivated worldwide (Wendel and Cronn, 2003). Commonly called upland cotton (*G. hirsutum* L.) is an economically important multi-purpose crop grown for its fibre (for use in textiles), seed oil (for use as a food ingredient) and for a protein rich meal (for use as an animal feed) (Liu *et al.*, 2009, 2012). At global scale, India, USA and China are the largest cotton producers, and during 2019–20 they produced 6.42, 5.93 and 4.34 million metric tons of cotton fibre, respectively (https://www.statista.com/statistics/263055/cotton-production-worldwide-by-top-countries). In the USA, Southern states are the main producers of cotton, and the United States cotton crop in 2018 and 2019 was valued at around $6.38 and $6.01 billion US dollars, respectively (https://www.nass.usda.gov).

Apart from the fibre or lint, cottonseed is a source of edible oil, and after separation of the oil, the residual meal or cake is used as a feed for ruminant livestock or as an organic fertilizer. Cottonseed normally contains about equal amounts of protein (20–25%) and oil (20–25%) by weight, and cottonseed oil represents a standard in the vegetable oil industry with a composition of about 26% palmitic (16:0), 2% stearic (18:0), 15% oleic (18:1) and 55% linoleic (18:2) acids (Jones and King, 1996; Liu *et al.*, 2012).

Cottonseed oil also contains relatively high levels of tocopherols. The total tocopherol content of crude cottonseed oil is about 1000 ppm with the α- and γ-forms accounting for 41% and 58%, respectively, of the tocopherol content. In addition to being metabolically important, the tocopherols also serve as strong antioxidants, contributing to the shelf life of cottonseed oil and preserving the freshness of products fried in the oil (Ghazani and Marangoni, 2016; Harwood *et al.*, 2017). Like most oils

derived from most dicot oilseeds, cottonseed oil has little or no tocotrienols, the vitamin E tocochromanol class with unsaturated side chains. Vitamin E is a generic term that refers to any of the eight naturally occurring forms of tocochromanols (i.e. α-, β-, γ- and δ-tocopherol and -tocotrienol). Tocopherols occur naturally in all plants, algae and some cyanobacteria; whereas tocotrienols occur naturally in most monocot and some dicot seeds, mostly in cereal grains and in palm fruits and seeds (Aggarwal *et al*., 2010; Fritsche *et al*., 2017; Gutbrod *et al*., 2019; Horvath *et al*., 2006; Mene-Saffrane, 2018; Morales *et al*., 2014). Tocotrienols differ structurally from tocopherols by the presence of three trans oriented double bonds in the hydrocarbon tail, whereas in tocopherols the aliphatic tail is fully saturated (Kamal-Eldin and Appelqvist, 1996). Tocopherol and tocotrienol biosynthesis differs only in the isoprenoid substrate in the initial homogentisate prenylation reaction: tocopherol biosynthesis uses a saturated phytyl diphosphate (PDP) substrate, while tocotrienol biosynthesis uses an unsaturated geranylgeranyl diphosphate (GGDP) substrate (Figure 1; Cahoon *et al*., 2003; Yang *et al*., 2011). These reactions are typically catalyzed by enzymes with distinct specificities for each isoprenoid substrate.

Because of their antioxidant activity (Fritsche *et al*., 2017; Gutbrod *et al*., 2019; Mene-Saffrane, 2018; Shahidi and Costa de Camargo, 2016), tocochromanols can protect plant cells against oxidative stresses arising from the breakdown of polyunsaturated fatty acids in seed oils. Moreover, the antioxidant activities of tocotrienols contribute to the nutritive value of food products and animal feeds derived from cereal grains. Medicinally, tocotrienols have been reported to possess powerful neuroprotective, anti-cancer and cholesterol lowering properties that are often not exhibited by tocopherols (Sen *et al*., 2006). Tocotrienols are found in many monocot cereal grains, for example, in wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), rye *(Secale cereale* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), etc., and are found in palm plants, such as oil palm (*Elaeis guineensis* Jacq.) and coconut (*Cocos nucifera* L.) (Horvath *et al*., 2006). Because of their health-promoting properties, tocotrienols are commercially produced as nutraceutical products from deodorizer distillates of rice bran and palm oils (Aggarwal *et al*., 2010; Cahoon *et al*., 2003; Falk and Munne-Bosch, 2010; Munne-Bosch and Alegre, 2002; Sen *et al*., 2006).

In monocots, homogentisic acid geranylgeranyl transferase (HGGT) catalyzes the committed step of tocotrienol biosynthesis (Cahoon *et al*., 2003; Yang *et al*., 2011). HGGT encoding cDNAs have been isolated from barley, wheat and rice seeds. Heterologous expression of the barley HGGT in *Arabidopsis thaliana* leaves resulted in a 10- to 15-fold increase in overall tocochromanol levels compared to non-transformed plants (Cahoon *et al*., 2003). Overexpression of the barley HGGT in corn seeds was shown to result in a sixfold increase of tocochromanols (Cahoon *et al*., 2003). Most recently, Konda *et al*. (2020) genetically engineered soybeans for seed-specific heterologous expression of a barley HGGT, either alone or in combination with a soybean γ-tocopherol methyl transferase (γ-TMT). The transgenic soybean

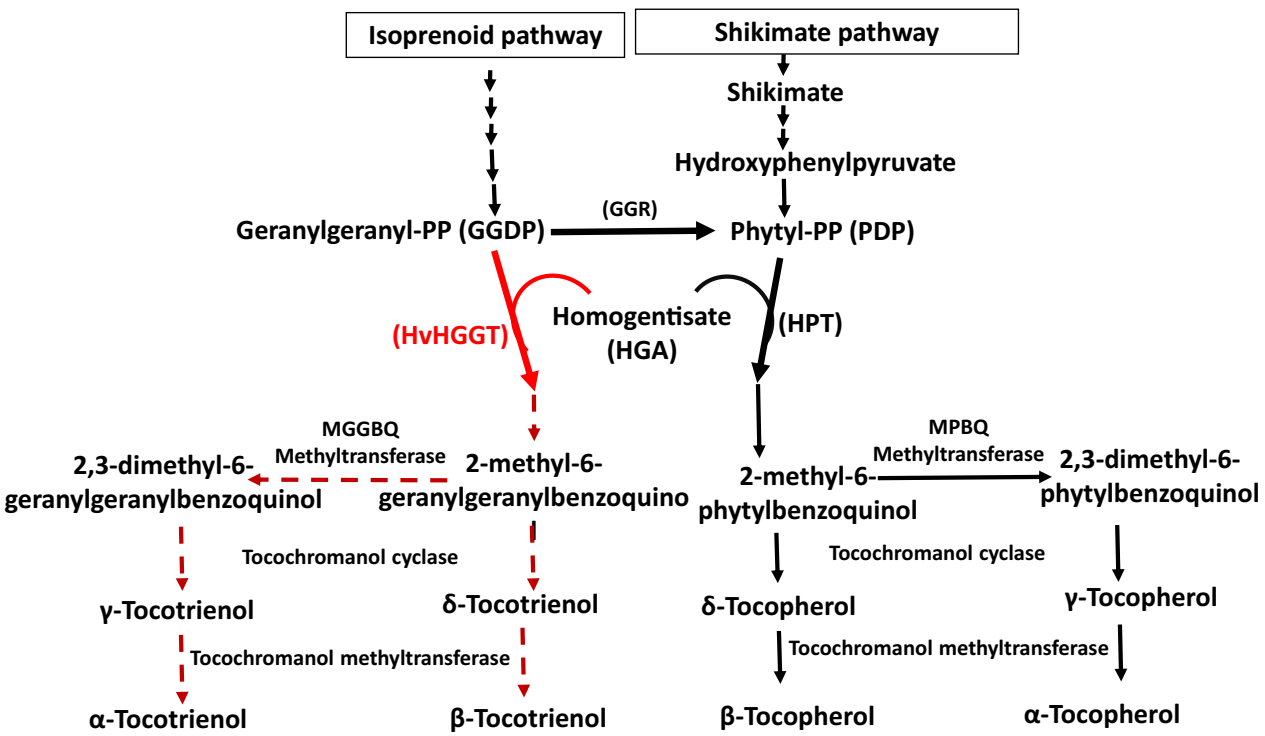

Figure 1 Biosynthesis pathway for tocopherols and tocotrienols engineered into cotton plants. Metabolic strategy to direct tocotrienol accumulation in cotton seeds by expression of barley homogentisate geranylgeranyl transferase (HvHGGT) (shown in red broken arrows). Homogentisic phytyltransferase (HPT) and homogentisic geranylgeranyl transferase (HGGT) catalyze the committed step of tocopherol and tocotrienol biosynthesis, respectively. Tocopherol biosynthesis occurs naturally in cotton (shown in dark solid arrows). HGA, homogentisate (homogentisic acid); HvHGGT, *Hordeum vulgare* (Hv) homogentisate geranylgeranyltransferase; HPT, homogentisate phytyltransferase; GGDP, geranylgeranyl diphosphate; MGGBQ, 2-methyl-6-geranylgeranyl-benzoquinol; GGR, geranylgeranyl reductase; PDP, phytyl diphosphate; MPBQ 2-methyl-6-phytyl-benzoquinol; (Figure based on Cahoon *et al*., 2003; Konda *et al*., 2020; Morales *et al*., 2014 and this study).

lines accumulated eightfold to 10-fold higher levels of total tocochromanols, principally as tocotrienols, with little effect on seed oil or protein concentrations. These detailed investigations demonstrate the possibilities for enhancing the vitamin E content in crops through redirection of metabolic flux by HGGT transgene expression (Cahoon *et al*., 2003; Konda *et al*., 2020).

Here, we developed transgenic cotton lines with altered seed biochemistry and enhanced nutritional value through embryo-specific (i.e. kernel-specific) expression of *HvHGGT* (Figure 1). In this manuscript, we have: (1) tested the hypothesis that the seed-specific expression of HGGT gene would result in production and accumulation of tocotrienols in cotton embryos; (2) studied the overall effect of transgene on plant growth, development, cottonseed composition; (3) evaluated the antioxidant properties conferred to transgenic seeds; and (4) examined the distribution of tocochromanols (i.e. sites of storage) within the mature cotton embryos. Collectively, we have demonstrated a heritable increase in vitamin E content in cottonseed, especially accumulation of tocotrienols, offering a novel cottonseed oil with enhanced antioxidant characteristics and extractable yields of health-promoting tocotrienols.

Results and discussion

Genetic characterization of HvHGGT transgenic cotton lines

In the present study, several independent *G. hirsutum* var. Coker 312 transgenics expressing HvHGGT enzyme were generated by *Agrobacterium*-mediated genetic transformation, and two events (HGGT1 and HGGT2) were advanced through to a field trial for further characterization. The T-DNA portion of the binary construct used for transformation is shown in Figure S1. The *HGGT* coding sequence was under control of the *NAPIN* seed-specific promoter, and plant selection was afforded by including kanamycin resistance. Cotton transformation is a time consuming, largely genotype-dependent process and involves co-cultivation of *Agrobacterium* with embryogenic cell lines followed by regeneration of plants by somatic embryogenesis (Figure 2). Callus was generated from cotyledon explants of aseptically germinated seedlings (Figure 2a), and these calli were sub-cultured, first, on solid media (Figure 2b) and, then, in liquid media (Figure 2c) until they became embryogenic. Following co-cultivation with *Agrobacterium tumefaciens*, embryogenesis was induced under selection (Figure 2d), The T_0 regenerated plantlets are matured (Figure 2e–f) and, finally, are transferred to the greenhouse for seed production (Figure 2g–k). Primary transgenics were confirmed by PCR analysis of genomic DNA (Figure S3). Three of six regenerated plants showed no DNA amplification and were eliminated assuming that the transgene was not present. The transgenic plants were advanced from T_0 to T_4 generation, and all the experiments confirmed HGGT transgenics at each seed/plant generation. PCR analysis of genomic DNA of transgenic plants, from T_0 to T_2 confirmed inheritance of the *HGGT* transgene (Figure S3). No HGGT specific 593 bp DNA band was found in any of the non-transgenic Coker 312 control plants.

Photosynthesis rates and yield characteristics in HGGT transgenic plants

Numerous environmental factors (e.g. sunlight, temperature, soil moisture, atmospheric CO_2 concentration, etc.) and plant characteristics (e.g. phyllotaxy, leaf shape, area of leaf, stomatal conductance, etc.) influence the physiology and photosynthesis rates of crop plants. Photosynthesis rate and crop yield are closely related to the net photosynthetic assimilation of CO_2 throughout the plant growing season (Friend and Helson, 1976; Zelitch, 1982). In the present study, 45 PCR-confirmed T_2 transgenic plants and five non-transgenic Coker 312 plants were assessed for their photosynthesis rates with the Li-Cor LI-6400XT system. The photosynthetic rates for the terminal (crown layer top) leaves from early vegetative to fruiting growth stage were very similar (Figure 3a). A 25–50% reduction in photosynthetic rate was observed for fully expanded lower leaves when compared to the top leaves from the 6th to 9th week of plant growth (Figure 3b, c). In comparison to the non-transgenic Coker 312 plants, the transgenic plants showed similar rates of photosynthesis through the nine weeks of measurement and through to boll maturity (Figure 3a–c). Thus, the *HGGT* transgene had no apparent influence on the photosynthetic performance in the leaf canopy, as might be expected for a seed-specific trait.

As HGGT transgene was under control of a seed-specific promoter, several seed-related yield parameters were compared for the 50 plants. As for the photosynthetic rate measurements, both transgenic lines performed similar (or better) in terms of average yield components (Figure 4). Although there were statistically greater numbers of bolls per plant for both of the transgenic plant lines compared with the non-transgenic Coker 312 plant line, this did not translate into statistically higher plant yields of seed cotton (fibre plus seed), fibre or seed (Figure 4a). Yields averaged higher for the HGGT1 and HGGT2 transgenic plants but the differences were not significant, likely due to more variable boll sizes in the transgenic plants compared with the Coker 312 plants. Additionally, average seed size also was not different between the HGGT transgenics and Coker 312 controls (similar average numbers of seeds per 3 g samples, Figure 4b). The average seed oil and protein contents were also similar among these plant lines. Therefore, the presence of the HGGT gene had no negative impact on the important economic yield parameters of fibre, seed oil or seed protein in these transgenic cotton plants.

Vitamin E production in transgenic cotton embryos

The *HGGT* transgene was designed to be expressed in cotton embryos and to direct the synthesis of tocotrienols. Table 1 summarizes the HPLC measurements of tocochromanols for generations of the HGGT1 and HGGT2 (T_1 embryos through T_4) compared with the non-transgenic Coker 312 embryos (see Figure S4 for representative HPLC traces). Embryos of both HGGT1 and HGGT2 accumulated high quantities of γ-tocotrienols (134.7–743.1 μg/g, dry embryo weight) and small approximately equal amounts of α-tocotrienol (2.4–16.3 μg/g) and δ-tocotrienol (3.3–39.5 μg/g). β-Tocotrienol was completely absent. No tocotrienols were found in the non-transgenic Coker 312 embryos, although α-tocopherol (41.5–153.5μg/g) and γ-tocopherol (46.1–231.7 μg/g) were observed. In the *HGGT* transgenic embryos, the α-tocopherol (25.4–142.8 μg/g) and γ-tocopherol (46.7–240.4 μg/g) ranges were similar to those observed in non-transgenic Coker 312 embryos. Very low amounts of δ-tocopherol were found in both the transgenic and non-transgenic Coker 312 embryos. Tocotrienols (α, γ and δ) contents of refined and bleached oils (RB oils) from field harvested transgenic seeds were about twofold to threefold higher than the samples analyzed from embryo oils. Similarly, the tocopherol contents of

Figure 2 Visual stages for *G. hirsutum* explant culture to produce callus and transgenic plants on MSM media through somatic embryogenesis and cotton transformation with pKAN-NapinHvHGGT binary vector. (a) *Explant* tissue culture of expanded cotyledon (one month old) producing calli, (b) Cotyledonous *explant* tissue sub-culture (3 month old) producing proliferated calli, (c) Two-month-old Somatic Embryogenic Cells (SECs) in MSM liquid media, (d). Post co-cultured cotton somatic embryogenic cells (*Agrobacterium* + SECs) developed into transgenic embryos on kanamycin (50 μg/mL) selection media, (e–f). T0 transgenic plants with differentiated root and shoot system at 2-4 leaf stage on selection media in magenta box, (g). Mature HGGT T0 plant in greenhouse with fully bloomed flowers, (h) Open flower of T0 plant −1DPA, I. Developing cotton boll (10 DPA) of T0 plant, J. Flower of T1 plant at anthesis and K. Developing cotton boll of T1 plant (20 DPA). (DPA - Day Post Anthesis).

both transgenics and non-transgenic Coker 312 were about 2 times higher as well.

In many higher plant seeds, α-tocopherol is often a minor component although there are exceptions. Of the different forms of tocochromanols, α-tocopherol has the highest vitamin E activity (DellaPenna and Pogson, 2006). Smith and Creelman (2001) analyzed 18 genotypes (varieties) of *G. hirsutum* seeds from different locations and harvest years for a genotype X environment study of tocopherol content. Averages of 200–220 μg/g total tocopherol content with 125–185 μg/g in the alpha form, 1.8–2.0 μg/g in the delta form, and 40–57 μg/g in the beta/gamma forms were observed. α-Tocopherol was positively influenced by high temperatures during the growing season (Smith and Creelman, 2001). These ranges of cottonseed α- and γ-tocopherols were somewhat different from the control Coker 312 genotypes quantified here, where the α- and γ- tocopherol levels were approximately equal or where γ-tocopherols were higher than α-tocopherol. These differences may arise from genetic, environmental or analytical differences. Nonetheless, α- and γ-tocopherols were the two most abundant tocopherols measured in our study, and the *HGGT* transgenic events were compared appropriately to the control Coker 312 genotype from which they were derived.

HPLC analysis of the *HGGT* transgenic embryos from T_1 through T_4 and the extracted refined and bleached oils (RB oils) from field-grown seeds showed the production and accumulation of tocotrienols in addition to tocopherols (Table 1). Introduction of the transgene *HvHGGT* gene into Coker 312 background resulted in slightly imbalanced accumulation of the α- and γ-tocopherols, perhaps due to the activity of the HGGT enzyme and the overall tocotrienol production. In the transgenic seeds, in comparison with wild type Coker 312 seeds, there was a considerable shift in the γ-tocopherol production, with almost threefold increase in accumulation of total tocochromanols and with a slightly reduced accumulation of α-tocopherol. In HGGT transgenic embryos, α- and γ-tocopherols were detected in roughly equal quantities. However, due to the additional production and accumulation of tocotrienols, there was a heritable, about threefold increase in total vitamin E content in the transgenic seeds compared to non-transgenic, Coker 312 seeds (Table. 1).

Studies in *Arabidopsis thaliana* with a series of VTE (Vitamin E deficient) mutants showed that tocopherol biosynthesis is mostly chlorophyll-dependent and that the tocopherol biosynthesis primarily originates in both leaves and seeds (Vom Valentin *et al*., 2006; Dorp *et al*., 2015; Zhang *et al*., 2015), although this is less clear in seeds (like cotton seeds) that lack chlorophyll. Horvath *et al*. (2006) investigated tocochromanol content and distribution in more than 80 plant species belonging to a wide range of mono- and dicotyledonous flowering plant families. All

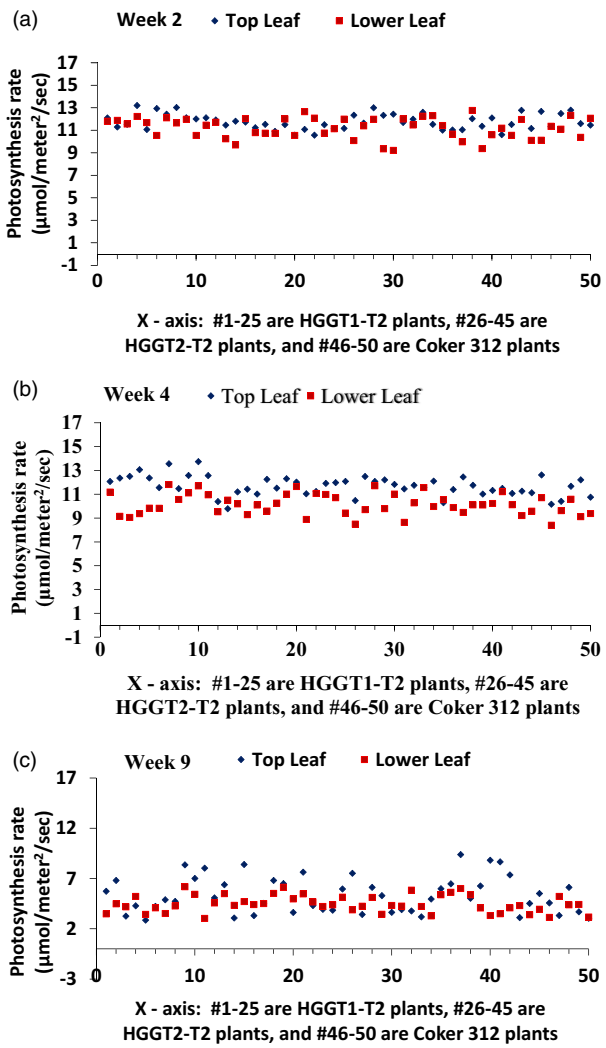

Figure 3 Photosynthesis rates in transgenic (T2) and Coker plants over 9 weeks time period which covered from vegetative (week 2, Figure 3a), flowering and fruiting (week 4, Figure 3b) and full plant maturity to early senescence (week 9, Figure 3c) stages. Plant #1-25: T2 progeny of HGGT1 (T1), plant #26-45: T2 progeny of HGGT2 (T1) and plant #45-50: non-transgenic Coker 312. Average photosynthesis rates were measured at early vegetative stage of one-and-half-month-old plants showing vigorous growth and development. Photosynthesis rate measured at week 2 showed fairly uniform rates in top layer/terminal leaf (blue data points) and lower layer (red data points) of leaves of transgenic (T2) and non-transgenic Coker 312 plants. (b) Plants continued to show higher rates of photosynthesis in upper leaves, but a minor relative decrease in lower leaves, among all the plants during early stages of flowering and fruiting/boll formation. (c) Photosynthesis rates were lower and more variable in the lower layer of leaves as the bolls fully matured and leaves became yellow (early signs of senescence).

the species investigated contained tocopherols irrespective of the source of the plant tissue that included leaves, seeds, flowers, latex and root tissue. Tocotrienols were restricted to non-photosynthetic tissues of seeds, fruits and latex, suggesting that tocotrienols accumulate at sites where chlorophyll synthesis and phytyl-PP levels are lowest. Although biosynthetic enzymes responsible for vitamin E are mostly found in chloroplasts and chromoplasts, maximum accumulation of vitamin E occurs during transition of chloroplasts to chromoplasts, that is, in the early onset of senescence of green leaves and floral tissues and the filling of seeds (embryo and endosperm maturation) (Hunter and Cahoon, 2007). In cottonseeds, which are non-photosynthetic and are not pigmented during development, the regulation of the biosynthesis of tocopherols and the involvement of chlorophyll as a source of phytol is unlikely (Fritsche *et al.*, 2017; Gutbrod *et al.*, 2019). Instead, it is likely that geranylgeranyl diphosphate (GGDP) is the biosynthetic precursor of the phytol side chain for tocopherol synthesis and that this pool of GGDP is utilized for tocotrienol synthesis as well as tocopherol synthesis in the HGGT-expressing transgenic cottonseeds (Fritsche *et al.*, 2017; Gutbrod *et al.*, 2019). However, the overwhelming accumulation of γ-tocotrienol with little formation of α-tocotrienol suggests that the methyltransferase activities may become limiting with the higher levels of tocochromanol synthesis in these transgenic tissues. Nonetheless, this is the first report of tocotrienol production in cottonseeds, and the overall threefold increase in vitamin E content may offer improved nutritional profile for cottonseed-derived products.

Localization of tocochromanols in HGGT transgenic embryos

It is possible that the deficient methylation of tocotrienols in the transgenic embryos results from a limitation of methyltransferase activity due to the much higher overall tocochromanol production. Alternatively, differences in tocochromanol compartmentalization in the transgenic cottonseed embryos might contribute to this reduction in methylation of the newly- introduced tocotrienol class. Tissue distributions of lipid metabolites in cottonseeds can be visualized by matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) imaging (Horn and Chapman, 2014a, 2014b; Horn *et al.*, 2012; Horn *et al.*, 2014; Sturtevant *et al.*, 2017). This technique was used to visualize and compare the distribution of tocochromanols and other lipophilic metabolites in the HGGT transgenic cotton embryos. MALDI-MS images localizing vitamin E constituents in sections of embryos are shown in Figure 5A (a–f) for Coker 312, in Figure 5B (a–f) for HGGT1 embryos and Figure 5C (a–f) for HGGT2 embryos. Coker 312 embryos showed the presence of tocopherols only, as is indicated by red pixels in Figure 5A (b) and Figure 5A (d). As expected, no tocotrienols were observed in the Coker 312 embryo sections above the background level (green). MS signals for δ-tocopherols were too low in abundance to image well. In contrast, the transgenic embryos showed strong signals for the presence of tocopherols as well as γ-tocotrienols [Figure 5B (a–e) and Figure 5C (a–e)]. For comparison, gossypol (a reproductive toxin in cottonseed) distribution was also analyzed and shown to localize to discrete gland regions in all embryos [Figure 5A (f), 5B (f) and 5C (f)]. Distributions and abundances were plotted as mol % values on a colour scale from green (low) to red (high) and were scaled to individual molecular species to aid in visualizing the distribution between tissues.

MALDI-MS image analysis showed that α-tocopherols were mostly localized to the cotyledonary tissues and γ-tocopherols were relatively enriched in embryonic axis tissues of both Coker 312 and the transgenic HGGT embryos lines, revealing a surprising spatial compartmentalization of these two tocopherol species. In contrast, newly-generated γ-tocotrienol was

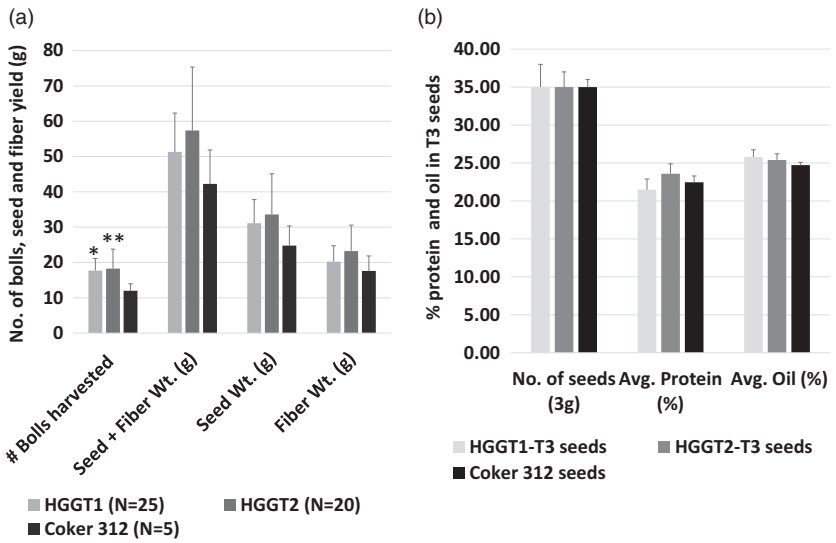

Figure 4 Yield performance of HGGT T2 plants in comparison to non-transgenic Coker 312 (a). Comparative yield analysis of cotton boll numbers, and total weight of seed and fibre harvested from HGGT (T2) lines and non-transgenic Coker 312 plants. Average yields from 25 T2 plants of HGGT1, 20 plants of HGGT2 and 5 plants of Coker 312. Statistically significant (**P = 0.003 and *P = 0.031) higher number of cotton bolls in T2 plants in comparison to Coker 312 were recorded. Seed and fibre yields, assessed together or separately, after ginning, were higher in T2s than Coker 312. (b) Seed index, average protein and oil content in HGGT - T3 and Coker 312 seeds. T3 seeds of T2 plant #1-25 (HGGT1), and T2 plant #26-45 (HGGT2) and plant #46-50 non-transgenic Coker 312 seeds showed no significant difference in seed oil or protein content. Time-domain NMR oil and protein data included three measurements per plant, average of 75 measurements for HGGT1, 60 measurements for HGGT2 and 15 measurements for Coker 312. Seed index (number of seeds per 3gm) as an estimate of seed size, was identical across transgenic and Coker 312 seeds with about 35 seeds per 3 gram.

distributed relatively uniformly throughout all transgenic embryo tissues. Also, the expression of *HvHGGT* in the transgenic embryos did not disturb the distribution of the α- or γ-tocopherols, as shown in Figure 5B (b and d) and Figure 5C (b and d). Similar to the δ-tocopherols, the δ-tocotrienols were too low in abundance or failed to ionize well enough to visualize by this method. Regardless of genotype, all seed tocochromanols were mostly excluded from the gossypol glands, reflecting a metabolic dedication to secondary metabolites in gland cells.

The heterogeneity of the α-tocopherol (cotyledonary tissues) and γ-tocopherol (embryonic axial tissues) in both the transgenic and Coker 312 plant lines may suggest that the methyltransferase needed to add the last methyl group to α-tocopherol is expressed in a tissue-specific manner, within the cotyledonary tissues where it converts most of the γ- tocopherol to α-tocopherol. On the other hand, the apparent lack of tissue-specific heterogeneity of the tocotrienols in the transgenic lines showed that the expression of the transgene was universal throughout the embryo and was not restricted to particular tissues. This suggests that HGGT can access the GGDP substrate pool for tocotrienol production, and the initial methyltransferase (VTE3) and cyclase (VTE1) enzymes that participate in the production of γ-tocopherol will also utilize the unsaturated metabolites. However, the corresponding methyltransferase that might be expected to produce α-tocotrienol (VTE4) does not appear to utilize γ-tocotrienol efficiently to produce α-tocotrienol, despite the colocalization of the methyltransferase and γ-tocotrienol in the embryo's cotyledonary tissues. It is likely that with the much higher amounts of overall tocochromanol produced, the methyltransferases become limiting and there is consequently less conversion of γ-tocotrienol to α-tocotrienol than might be anticipated in the cotyledonary tissues of the transgenics.

Increased tocotrienol content and oxidative stability of refined and bleached oils from field-grown cotton plants

Tocochromanols are well known for their antioxidant properties, and we undertook a field-based seed increase to test seed extracts for antioxidant activity and extracted cottonseed oil for oxidative stability. T_4 embryos harvested from the field-grown HGGT1 and HGGT2-T3 plants as well as from the non-transgenic Coker 312 plants were subjected to tocochromanol analysis (Figure 6; Table 1). In comparison to T_3 embryos (from greenhouse-grown plants), the total tocotrienol contents were increased considerably in T_4 the field-grown embryos. In the T4 embryos, the δ-tocotrienol content ranged from 37 to 40 μg/g, whereas it was 8 to 10 μg/g in T_3 embryos. Similarly, the β/γ-tocotrienol content in T_4 seeds ranged from 727 to 743 μg/g, whereas β/γ-tocotrienol content in T_3 embryos were around 300 μg/g. Consequently, the overall tocotrienol content in T_4 embryos of two transgenic lines more than doubled (767 and 780 μg/gm) in comparison to the total tocotrienol content recorded from the greenhouse harvested embryos (321–326 μg/g). Differences in tocopherol content were also observed between field-grown (T_4) and greenhouse-grown (T_3) embryos. Total tocopherol contents in *HGGT* embryos harvested from the field were 375 to 383 μg/g, while the non-transgenic Coker 312 embryos were ~376 μg/g, all of these values being substantially higher than the greenhouse harvested seeds (230 and 239 μg/g for the HGGT embryos and ~267 μg/g for the Coker 312 embryos). The increase in both tocopherol and tocotrienol content in the field samples was likely due to exposure of the plants to enhanced natural growth conditions (light and temperature). Production of tocopherols has been noted to be positively

Table 1 Analysis of Vitamin E [Tocotrienol + Tocopherol] content of HGGT (T1 through T4) transgenic and Coker 312 (non-transgenic) embryos (Excel file uploaded separately)

Plant ID/seed generation	No. plants	No. replicates	Tocotrienol constituents ± SD (μg/g)			Tocopherol constituents ± SD (μg/g)			Total T3 ± SD (μg/g) (α + γ+δ)	Total Toco ± SD (μg/g) (α + γ+δ)	Total Vitamin E ± SD (μg/g) (T3 + Toco)
			α-T3	γ-T3	δ-T3	α-Toco	γ-Toco	δ-Toco			
[1]Coker 312 embryos	1	n = 1	ND	ND	ND	48.74	46.05	2.54	ND	97.33	97.33
[2]HGGT1 - T1 embryos	1	n = 1	2.89	134.68	3.29	41.54	46.68	1.68	140.86	89.9	230.76
[3]HGGT2 -T1 embryos	1	n = 1	2.44	163.04	5.63	25.44	59.08	0.72	171.11	85.24	256.35
[2]HGGT1 - T2 embryos	5	n = 5 (1 from each plant)[†]	5.12 ± 0.41	164.62 ± 15.12	3.82 ± 0.65	27.53 ± 2.73	69.25 ± 5.31	2.68 ± 0.59	173.56 ± 15.37	99.45 ± 6.33	273.10 ± 17.89
[3]HGGT2 -T2 embryos	4	n = 4 (1 from each plant)[†]	4.05 ± 0.92	173.69 ± 18.47	3.92 ± 0.72	28.34 ± 1.98	68.80 ± 3.43	2.68 ± 1.19	181.66 ± 18.48	99.82 ± 3.75	281.48 ± 18.27
[1]Coker 312 embryos	5	n = 15 (3 from each plant)[†]	ND	ND	ND	153.48 ± 9.54	113.49 ± 13.71	ND	ND	266.97 ± 8.39	270.43 ± 13.39
[2]HGGT1 - T3 embryos	25	n = 75 (3 from each plant)[†]	16.32 ± 3.94	300.06 ± 21.63	9.98 ± 4.82	116.01 ± 12.40	122.68 ± 18.12	ND	326.36 ± 23.73	238.69 ± 11.97	565.05 ± 28.41
[3]HGGT2 -T3 embryos	20	n = 60 (3 from each plant)[†]	13.93 ± 3.89	299.70 ± 28.73	7.62 ± 2.29	116.72 ± 9.94	112.98 ± 10.63	ND	321.25 ± 29.65	229.70 ± 11.41	550.95 ± 23.83
[1]Coker 312 embryos	Field trial bulk	n = 4	ND	ND	ND	136.25 ± 19.09	231.7 ± 10.33	7.75 ± 1.19	ND	375.71 ± 24.94	375.71 ± 24.94
[2]HGGT1 - T4 embryos	Field trial bulk	n = 4	ND	743.08 ± 6.53	37.11 ± 9.50	142.81 ± 6.87	217.85 ± 7.72	13.9 ± 1.86	780.19 ± 11.93	374.56 ± 6.81	1154.75 ± 11.22

Table 1 Continued

Plant ID/seed generation	No. plants	No. replicates	Tocotrienol constituents ± SD (μg/g)			Tocopherol constituents ± SD (μg/g)			Total T3 ± SD (μg/g) (α + γ+δ)	Total Toco ± SD (μg/g) (α + γ+δ)	Total Vitamin E ± SD (μg/g) (T3 + Toco)
			α-T3	γ-T3	δ-T3	α-Toco	γ-Toco	δ-Toco			
[3]HGGT2 -T4 embryos	Field trial bulk	n = 4	ND	727.38 ± 18.13	39.52 ± 8.35	135.38 ± 6.83	240.35 ± 4.70	6.81 ± 0.61	766.9 ± 20.81	382.54 ± 6.18	1149.44 ± 25.72
[1]Coker 312 seed oil[‡]	Field trial bulk	n = 5	ND	ND	ND	379.48 ± 6.06	574.97 ± 8.13	ND	ND	954.45 ± 13.57	954.45 ± 13.57
[2]HGGT1 - T4 seed oil[‡]	Field trial bulk	n = 5	21.36 ± 0.76	1571.25 ± 22.00	40.68 ± 1.13	308.00 ± 1.62	512.25 ± 5.85	ND	1633.30 ± 22.16	820.25 ± 6.60	2453.55 ± 28.45
[3]HGGT2 - T4 seed oil[‡]	Field trial bulk	n = 5	20.75 ± 1.43	1493.19 ± 18.66	39.38 ± 1.03	288.93 ± 3.64	530.69 ± 5.49	ND	1553.32 ± 19.41	819.63 ± 9.05	2372.95 ± 28.30

[1]Coker 312 is non-transgenic, [2]HGGT1 and [3]HGGT2 are transgenic lines. Coker 312 and HGGT -T1 through T3 embryos were from greenhouse-grown plants. Coker 312 seed oil and HGGT -T4 seed oils were from field trial harvest.

ND, Not Detected.

[†]Data presented are average value (±SD) when more than three replicates per line were analyzed. Each replicate included bulk of 10 embryos.

[‡]Refined bleached oil.

influenced by hot weather during the cotton crop growing season (Smith and Creelman, 2001).

Dehulled seeds of the three plant lines were hexane extracted and partially refined to produce RB oils, and the oil's tocochromanol compositions were determined (Figure 6b; Table 1). The total tocotrienol content (nearly all in the γ- form) was more than 1.5 mg/g in the HGGT-modified RB oils, and the total tocochromanol content of the HGGT oils was nearly three times that of control oil (~2.4 mg/g in HGGT oil vs 0.95 mg/g in Coker 312). This represents a sharp improvement over standard cottonseed oil in both the vitamin E content and composition. With annual global production of cottonseed of about 43 million metric tons in 2019, this represents a significant potential source of tocotrienols that could be recovered from the distillate fractions produced during crude cottonseed oil refining.

With the increased vitamin E content, it might be expected that the antioxidant properties of the seeds and RB oils of the HGGT-modified plants would be improved. Total tissue lysates prepared from the HGGT1 and HGGT2 embryos both showed nearly twice the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) antioxidant activity of the Coker 312 embryos (Figure 7a). In addition, RB oils showed a nearly 30% improvement in oxidative stability (Figure 7b). This indicates that transgenic cottonseed oil with elevated tocochromanol content provides extra stability and extended shelf life for the HGGT transgenic cottonseed oil.

Reduction in polyunsaturated fatty acid content is well known to improve the oxidative stability of vegetable oils, so we determined the triacylglycerol (TAG) composition by high resolution mass spectrometry of the HGGT-extracted seed oils (Figure S6). There was little change in overall TAG composition between the transgenic oils and the oil extracted from the Coker 312 seeds (Figure S6A). There was a slight increase in polyunsaturated fatty acids in the HGGT oils (e.g. TAG 54:5 and TAG 54:6 species that will mostly be enriched with 18:2 fatty acids), although these changes may not be statistically significant. Also, there was a trend in transgenic oils for a modest reduction in lower *m/z* TAG species that would be enriched in palmitic acid (16:0), such as TAG 50:2 (18:2/16:0/16:0) and TAG 52:2 (18:2/18:0/16:0). The distribution of several TAG species are shown in (Figure S5) and indicate that the slight changes in overall TAG composition (reductions in TAGs with 16:0, and increases in TAGs with 18:2) occurred mostly in the embryonic axis region. While these trends were not significant, the differences indicate an oil composition that would have more polyunsaturated fatty acids and less saturated fatty acids, and oils with more polyunsaturated fatty acid would be expected to be less oxidatively stable. Hence, improvement in the oxidative properties of the transgenic oils observed here cannot be accounted for by changes in fatty acid composition and are most likely accounted for by the changes in the tocochromanol levels of these oils.

The slight shift to longer chain and more unsaturated TAG in transgenic lines may represent an altered redox state within the cellular environment of embryos that is more favourable towards elongation and desaturation reactions. Additionally, the increased abundance of tocochromanols in the form of tocotrienols and their increased antioxidant capabilities may be protective of more unsaturated TAG species that would otherwise be more susceptible to oxidation.

The seed oils also were analyzed by high resolution mass spectrometry for tocochromanol composition (Figure S6B). Consistent with quantification by HPLC (Figure S7B; Table 1), the α- and γ-tocopherols observed in both Coker 312 and transgenic seed oils were of similar mol% proportions. No tocotrienols were found in non-transgenic Coker 312 seed oil, but were evident in the transgenic seed oils (Figures S6B and S7A), almost entirely as γ -tocotrienol. Tocochromanols were confirmed by MS/MS for characteristic fragment of the heterocyclic ring. For α-tocopherol and α-tocotrienol (Figure S7B,D) these fragments were *m/z* 165.13 across the heterocyclic ring and 205.16 between the heterocyclic ring and isoprenoid tail, and for γ-tocopherol and γ-tocotrienol (Figure S7C,E) these were *m/z* 151.11 across the heterocyclic ring and 191.15 between the heterocyclic ring and isoprenoid tail.

Conclusion

Here, we have introduced the coding sequence for a single enzyme (barley HGGT) into cotton embryos that redirects the vitamin E content and composition to favour the accumulation of tocotrienols. Overall vitamin E content increased threefold in seeds, with 60% being γ-tocotrienol. Given a widening array of health-promoting properties for tocotrienols, this new, enhanced profile of vitamin E in cottonseeds offers the potential for various nutraceutical applications. In addition, the improved oxidative stability of tocotrienol- containing cottonseeds and seed oils provides an added benefit of prolonged shelf life for cottonseeds and cottonseed-derived products.

Methods

Plant binary vector with *HvHGGT*-NPN sequences and cotton transformation

A plant transformation binary vector pKAN-NapinHvHGGT (16 125 bp) was constructed for heterologous expression of the barley HGGT in cotton seeds (Figure S1; Cahoon *et al*., 2003). This vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation, and colonies were selected on kanamycin + streptomycin LB (Luria-Bertani) media and confirmed by PCR to contain the plasmid. For transformation, cells were collected from an overnight liquid culture and grown in fresh medium to an optical density (OD_{600}) between 1.0 and 1.5. Cells were pelleted and put into co-cultivation medium (MSM, see below) at an optical density (OD_{600}) between 1.5 and 1.9.

Agrobacterium tumefaciens-mediated genetic transformation of cotton embryogenic cells

Cotton tissue culture and genetic transformation methods were modified from Leelavathi *et al*. (2004) and Rathore *et al*. (2006, 2015). MS (Murashige and Skoog, 1962) plant tissue culture medium was used containing 3% D-maltose (MSM medium) but with no additional nutrient supplements or hormones, such as kinetin, 2,4-dichlorophenoxy acetic acid (2,4-D), IAA, etc. (Fuller *et al*., 2011; Leelavati *et al*., 2004; Murashige and Skoog, 1962; Rathore *et al*., 2015; Trolinder and Goodin, 1987, 1988a, 1988b; Wilkins *et al*., 2004; Wu *et al*., 2008). In brief, the steps involved are outlined in Figure S2 and materials at representative stages are shown in Figure 2a–k. The cotton cultivar Coker 312 was used for tissue culture and transformation. Acid delinted seeds were surface sterilized and germinated on MSM media at 28 °C. Cotyledonary leaf segments (10–15 mm^2) from 5- to 7-day-old seedlings were excised and callus initiated and proliferated through sub-culture 3 or 4 times, once every 3 weeks, on MSM medium with KNO_3 (MSMK) at 28 °C. Rapidly growing pale

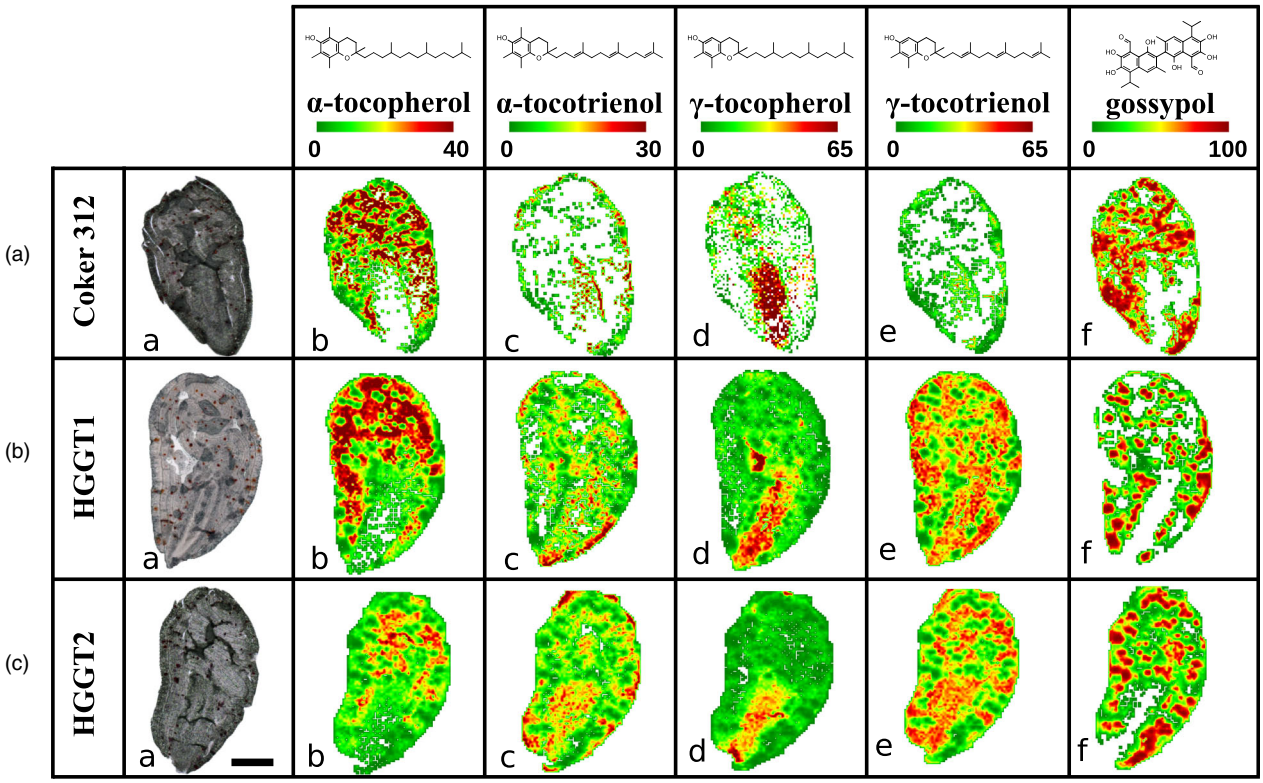

Figure 5 MS-MALDI imaging of tocochromanol distribution in longitudinal sections of mature cotton embryos: Coker 312 (A), HGGT1-T3 and HGGT2-T3 transgenics (B–C). Comparison of the tissue-specific distributions of tocochromanols in non-transgenic Coker 312 and HvHGGT expressing two independent events of transgenics embryos. Bright field microscope images of cotton embryos sections that were used for MS imaging are shown to the left. Delta- tocopherols and δ-tocotrienols either were too low to detect or did not ionize well enough to image. Mol per cent values of tocochromanol and gossypol content are plotted on a colour scale from green (low) to red (high); scale bars for each molecular species are set to individual values at the top. (scale bar = 1.5 mm). Arrows indicate cotyledonary tissues (top row-b) and embryonic axis (top row-d)

green calli were sub-cultured in MSM liquid medium (28 °C) to produce somatic embryogenic cell lines (SECLs). Approximately 2 mL volumes of the SECLs were co-cultured for 36 h with freshly prepared *A. tumefaciens* cells (cell density OD_{600} between 1.5 and 1.9) containing the plant expression vector pKAN-NapinHvHGGT (above) (Cahoon *et al.*, 2003) for *Agrobacterium*-mediated transformation. Following co-cultivation, SECLs were transferred to plant selection media with kanamycin (50 μg/mL) and carbenicillin (400 μg/mL) for embryogenesis and embryo development. Differentiated young plantlets were moved to magenta boxes containing MSM + kanamycin. After plantlets were about 5 cm tall, they were transplanted and acclimatized to soil in plant growth chambers. Transgenic plants at the 2 to 4 leaf stage were moved to 6 gallon plastic pots and grown to maturity in an air conditioned greenhouse. Greenhouse conditions for plant growth were set at 30 °C (day and night) temperature. During light conditions (16 h) the natural sunlight was supplemented with 1000 watt high-pressure sodium vapour lamps. When the bolls matured, seed cotton was harvested from each of the transgenic and non-transgenic (control) plants separately. After air drying for about one week at room temperature, the seed cotton was ginned with a 10-saw tabletop cotton gin to remove the lint. Seeds of these primary transformants were used for biochemical analysis and for progeny advancement.

Molecular characterization of transgenic plants

Transgenic plants from T_0 to T_3 were confirmed by PCR of genomic DNA. Genomic DNA was isolated from young leaves of the transgenics and non-transgenic Coker 312 seedlings using commercially available Plant DNAzol reagent (cat # 10978-021, Carlsbad, CA, www.invitrogen.com). DNA concentrations were determined by Nano spectrometry (reading at A600/A800) and adjusted to ~50 ng/μL with 0.5× TE (5 mM Tris-HCl, pH 7.5 and 0.5 mM EDTA, pH 8.0) buffer. PCR reactions (50 μL volume) contained 50 ng of genomic DNA, *Go Taq* Flexi DNA polymerase (1 unit) (Cat# M8296, Promega, Madison, WI), 1x Flexi buffer, 3 mM $MgCl_2$, 200 μM dNTPs and 0.5μM of each primer. PCR amplifications were carried out with a MJ Research PTC-200 Peltier Thermal Cycler with an initial denaturation temperature of 94°C for 2 min followed by 35 cycles of DNA amplifications at 92°C for 30 s (denaturation), 58 °C for 1 min (annealing) and 71 °C for 1 min (extension) followed by a final extension at 71 °C for 10 min. Endogenous actin gene specific primers (used as control) *Gh*. Actin-Forward- 5′-ACCGTGCCAATCTATGAACG-3′ and Reverse- 5′-ACGGAATCTCTCAGCTCCAA-3′ ~300 bp PCR fragment), and *HvHGGT* specific primers (Forward-5′-TGAG-GAAATCAGGGGAGATG-3′, and Reverse-5′-CGGCAGAGAAG-CAACACATA-3′- 593 bp PCR fragment) were used in DNA amplifications. PCR amplified DNA products were separated on

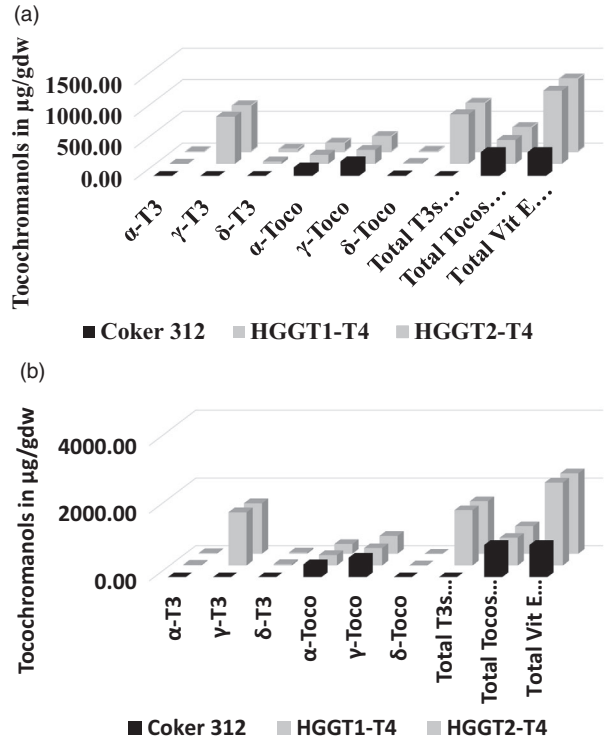

Figure 6 Quantitative analysis by HPLC of Vitamin E content in seeds (a) and extracted refined oils (b). Tocotrienols and tocopherols contents in microgram per gram dry weight (µg/g) of transgenic (T4) embryos in comparison to non-transgenic Coker 312 embryos (a). Seeds were randomly selected and dissected from field-grown plants. Data presented are average values of 4 replicate seed samples per genotype. Tocochromanols contents in refined bleached oils (b) were the average from five replicate samples per genotype. (Vit E, Vitamin E; T3, tocotrienols; Toco, tocopherol)

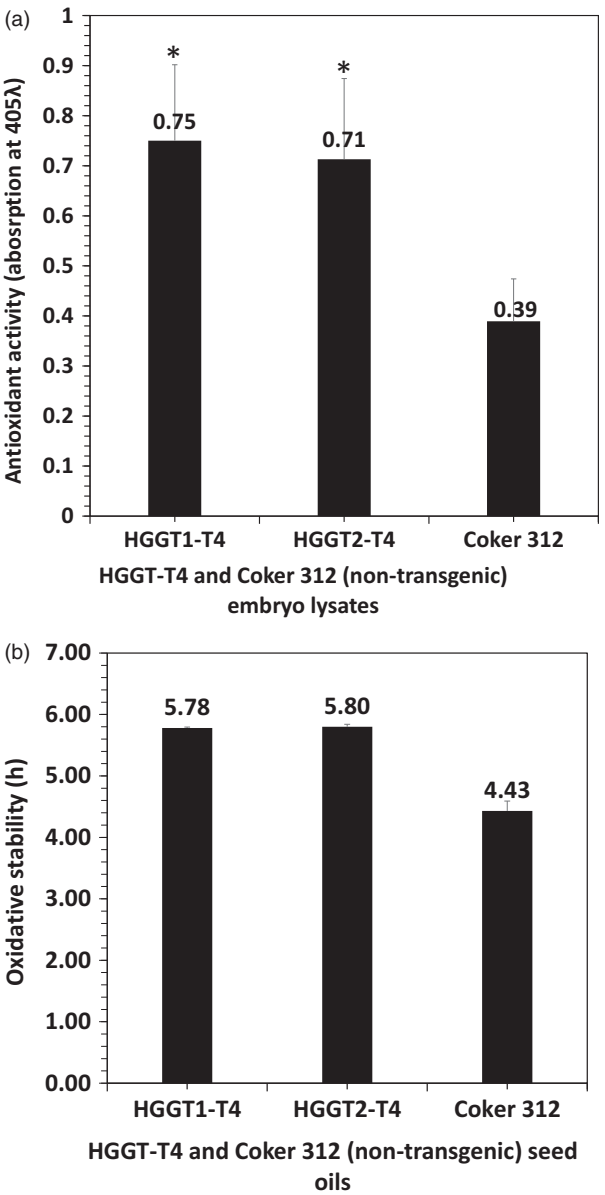

Figure 7 Antioxidant activity in seeds (a) and oxidative stability of refined oils (b). Antioxidant activity measured by ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) assays of embryonic tissue lysates showed improved antioxidant activity in HGGT-T4 compared to Coker 312 (non-transgenic) in (a). Oxidative stability index (in hours) of HGGT-T4 refined, bleached oils in comparison to Coker 312 (non-transgenic). Refined bleached oils were analyzed at Iowa Central Fuel Testing Laboratory, Fort Dodge, Iowa and measurements were made in triplicate. **P* = 0.03 (HGGT1) and **P* = 0.04 (HGGT2)

1% agarose, stained in ethidium bromide and visualized and photographed under UV light (Figure S3A-B).

Comparison of yield components and photosynthetic performance between HGGT transgenic and non-transgenic Coker 312 plants

To determine the effect of the HGGT transgene on yield components in cotton, a comparison of boll production, seed and total fibre yield were made among the two independent T_2 non-segregating transgenic events (25 T_2 sibling plants of HGGT1 and 20 T_2 sibling plants of HGGT2) and the non-transgenic Coker 312 background (5 plants). The parameters measured were the 1) average number of bolls per plant, 2) average weight of seed + fibre combined per plant, 3) average weight of ginned seed per plant and 4) average weight of ginned fibre per plant (Figure 4a). Data from transgenics as well as non-transgenic Coker 312 samples were subjected to statistical analysis using Student's *T*- test. The level of statistical significance was determined based upon the *p* value.

Photosynthetic performance (measured by CO_2 gas exchange; Figure 2a–c) was also monitored prior to and during boll set. At the outset, the 45 T_2 seedlings (progeny derived from two primary independent events above) were genotyped by PCR and moved to the greenhouse along with the five Coker 312 plants. All plants were grown in the greenhouse under uniform light, temperature and watering conditions. Greenhouse temperature was set at 30 °C (day and night). During the 16 h light conditions, the sunlight was supplemented with 1000 watt high-pressure sodium vapour lamps. Photosynthetic rate measurements (McGarry *et al*., 2016) were collected weekly starting from about two-month-old plants (showing vigorous vegetative growth) and continuing until boll maturity and early signs of senescence. Rates were measured (CO_2 exchange in micromole per metre square per second, µmol/metre^2/sec) using the portable

Li-6400XT System (Li-Cor Biosciences, Lincoln, Nebraska) fitted with a LED2x3 light source IRGA (Infra-Red Gas Analyzer) and leaf chamber fluorometer. As per manufacturer's recommendation, all photosynthesis readings were taken with constant instrument settings of CO_2 reference of 400, flow rate at 500 and photosynthetically active radiation (PAR) 300. Chamber block temperature and relative humidity were established by ambient air conditions and were 30 °C and ~60%, respectively. Plant leaves were allowed to acclimatize for about 2 min in the chamber before taking the readings. Photosynthesis rate measurements included 2 leaves per plant, the first representing the top crown layer (terminal leaf) and the second from fully grown/expanded middle layer of leaves. Three readings were taken per leaf at 30 s intervals of each measurement, and the average of three readings was used for each leaf.

Oil and protein content

Mature cotton bolls from greenhouse-grown T_0 through T_2 transgenic (HGGT1 and HGGT2) and Coker 312 non-transgenic plants were harvested by hand from individual plants. Bolls (with seed cotton) from each plant were pooled for ginning. Three grams of cottonseed (in triplicate) from each plant were placed into an 18-mm-diameter glass NMR tube and were conditioned for 2 h in 40 °C in a forced draft oven. Samples were subjected to quantification of oil and protein by low-field, time-domain ^{1}H Nuclear Magnetic Resonance (TD-NMR) spectroscopy in a Bruker minispec 20 following the method of Horn *et al*. (2011). Mean oil and protein values were calculated on a seed weight basis from the triplicate readings (Figure 4b).

Tocopherols and tocotrienols content analysis

Tocopherols and tocotrienols were quantified from extracts of dissected embryos for each successive generation of transgenic plants (T_0 through T_3) and the non-transgenic Coker 312 plants. Analysis of the tocochromanols (α, γ and δ) was performed by HPLC with fluorescence detection following the procedure, described by Konda *et al*. (2020). Excitation was at 292 nm and emission was monitored at 330 nm fluorescence (292 nm excitation/335 nm emission) Individual forms compounds were identified by comparing retention times with those of authentic standards. The tocochromanols were quantified by peak area and normalized to the internal standard 5, 7-dimethyltocol (Matreya) added at the time of extraction (Cahoon *et al*., 2003; Konda *et al*., 2020).

Seed increase for field-grown HGGT transgenic (T_3) and Coker 312 (non-transgenic) plants

T_3 seeds harvested from greenhouse-grown T_2 plants (originating from the two independent primary transgenic events) were bulked, acid washed and rinsed under tap water. The T_3 transgenic and non-transgenic Coker 312 seeds were transported under an APHIS-USDA Biotechnology Regulatory Services permit for Interstate Movement and Release Notification No. 18-102-101n (UNT-18-001) (regulated article-Upland cotton). Seed (~0.5 kg) for each of the two transgenic lines and the non-transgenic Coker 312 plant lines were planted and grown on ~1 acre farm plots near San Angelo, Texas (Tom Green County) and were maintained by Brandon Ripple, Ripple Agricultural Research, Inc. Mature bolls were hand harvested, and all of the collected seed cotton (approximately 20–25 kg per plant line) was ginned. A subsample of each devitalized seed used for biochemical analyses while the remainder (16–20 kg per plant line) of the T_4 seeds were used for pilot-scale solvent-extraction of the oil.

Pilot-scale oil extraction

Each of the bulked ginned seed samples was cracked in an 8″ Bauer plate mill, and the seed coats were separated on an 18″ Kason screen shaker fitted with #4 and #12 screens. The embryo pieces were then milled with an Alpine Z160 pin mill. Each sample was then extracted in 3 kg batches with 8 L of commercial hexane in a 20 L Buchi evaporator. The instrument was operated as an extractor by using a fluted flask to promote mixing and redirecting the condenser condensate back to the extraction flask. The extractions were conducted at 50 °C for 2 hr. At the end of the extraction period, the miscella (solvent and extracted oil) was separated from the defatted meal by vacuum filtering first with coarse creped filter paper. While on the filter, the retained meal washed with about 2 L of fresh hexane. The recovered miscella was then vacuum filtered a second time with finer #4 Whatman paper. The miscellas from the individual batches of each sample were combined, and the hexane was recovered with the Buchi evaporator operated at 70 °C under vacuum (~200 Torr).

The recovered crude oils were then refined and bleached (RB). The normal deodorization step typically conducted after bleaching was omitted, as this operation will remove some tocochromanols. To determine the amount of sodium hydroxide to use for refining, the free fatty acid levels of the oils were determined by titration against a standardized sodium hydroxide solution. Each oil was then refined at room temperature with 16° Bé sodium hydroxide (~11 % aqueous solution) added in an amount needed to neutralize the free fatty acids and provide a 0.35 % excess. The crude oil and sodium hydroxide were mixed for at room temperature for 30 min, then centrifuged at 8000 g for 20 min to separate the soapstock and impurities. The refined oil was then bleached with 4.7% AOCS Natural Bleaching Clay. Bleaching was conducted at 100 °C for 30 min under vacuum (~200 Torr). The spent bleaching earth was then separated by centrifugation at 8000 g for 20 min to yield between 1.7 and 1.8 litres of each RB oil.

Cottonseed oil testing

The RB oils were used in biochemical analysis and measurements of oxidative stability. Oxidative Stability Index measurements were done by a commercial service (Iowa Central Fuel Testing Laboratories, Fort Dodge, IA 50501) following the standard AOCS method EN15751:2014 (the modified Metrohm 873 Rancimat method to analyze the oxidative stability of biodiesel blends), (European Committee for Standardization EN 15751-Automotive Fuels-Fatty Acid Methyl Ester (FAME) Fuel and Blends with Diesel Fuel-Determination of Oxidation Stability by Accelerated Oxidation Method (2014). In addition, RB oil samples of each transgenic and Coker 312 plant line (5 replicates each) were also analyzed for tocochromanol content and triacylglycerol profiles. Student's *T*- tests were done to determine the statistical significance based upon the *P* value.

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay

The Zen-Bio ABTS antioxidant assay kit (cat# AOX-1, Zen-Bio, Inc., Research Triangle Park, NC) is used to determine the total antioxidant capacity of biological materials. The assay measures ABTS + radical cation formation induced by metmyoglobin and

hydrogen peroxide. Trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control inhibiting the formation of the radical cation in a dose dependent manner. The antioxidant activity in cells, tissues and natural extracts can be normalized to equivalent Trolox units to quantify the composite antioxidant activity present.

For ABTS antioxidant assay, cotton embryo extracts were prepared following the manufacturer's instructions, and extracts were prepared in triplicate for each of the two transgenic HGGT and the non-transgenic Coker 312 embryos. A total of 5 embryos per replicate were extracted. Samples were processed as per the protocol, and the absorbance readings were taken with a BMG Labtech Spectrostar *Nano* plate reader at a wavelength of 405 nm. Data from each of the transgenics as well as non-transgenic sample replicates were statistically tested using Student's *T*- test. Statistical significance was determined based upon the *p* value.

MALDI-MS imaging of tocochromanols in cottonseed *HvHGGT* embryos

For MALDI-MS imaging, the seed coats of 10 to 20 (per plant line) randomly selected mature dry seeds of HGGT transgenics (T_3) and Coker 312 (non-transgenic) embryos were removed by scalpel and forceps. Dissected embryos were then embedded in 10% porcine gelatin (Millipore Sigma, St. Louis, MO) prior to sectioning as has been described before (Sturtevant *et al*., 2017a, 2017b; Sturtevant *et al*., 2019). Embedded embryos were frozen at −80 °C for 16 h then equilibrated to −20 °C for ~5 days. Longitudinal tissue sections of 30 μm thickness were taken using a Leica CM1950 cryo-microtome (Leica Biosystems, Buffalo Grove, IL) and transferred to Superfrost Plus glass microscope slides (Fisher Scientific, Grand Island, NY) with CryoJane collection tape (Leica Biosystems). After sectioning, tissue sections were lyophilized for 3 h. Tissue sections chosen for mass spectrometry imaging were cut from microscope slides using a glass cutter and then coated with 2,5-dihydroxybenzoic acid (DHB) by sublimation as described previously (Hankin *et al*., 2007; Sturtevant *et al*., 2017a; Sturtevant *et al*., 2019). DHB coated sections were imaged using a MALDI-LTQ-Orbitrap-XL mass spectrometer (Thermo Scientific, Carlsbad, CA). The parameters used for MS imaging were as follows: a laser raster step size of 80 μm, 14 μJ laser energy, 10 laser shots per step, an m/z range collected from 375–1000. Data were processed using the open source software Metabolite Imager (Horn and Chapman, 2014a, 2014b). Tocochromanols were imaged as radical cations, as has been previously been found in other MS imaging experiments (Monroe *et al*., 2005) and were plotted as a coloured scale heat map representation of mol% intensity from green (low) to red (high) (Figure 5). As a contrast to tocochromanol distribution gossypol was also imaged as $[M + H - H_2O]+$ adducts [Figure 5A (f), 5B (f) and 5C (f)]. Triacylglycerol distributions were imaged as $[M + K]+$ adducts.

Mass spectrometric analysis of pressed cottonseed oil

Measurements of TAG and tocochromanol content from the solvent-extracted oils was done on a Waters Synapt G2 mass spectrometer. Approximately 3 mg of oil was dissolved in 1ml of $CHCl_3$ and then diluted 1:100 into a solution of $CHCl_3$, MeOH and 5 mM NH_4Ac (2:1:0.01 v/v/v). Diluted oil samples were directly infused into the mass spectrometer at a flow rate of 20 μL/min. The following parameters were used for the ionization source: temperature set to 80 °C, desolvation gas set to 500 L/h, and a capillary voltage set to 3 kV. The *m/z* range collected for analyzing TAG content was from 700 to 1000, and for analyzing tocochromanol content over 300 to 500. The identities of tocopherols and tocotrienols from non-transgenic Coker 312 cottonseed oil and HGGT transgenic cottonseed oil were confirmed by MS/MS, selecting for parent ion masses of 430.47 for α-tocopherol, 416.45 for β/γ-tocopherol, 424.43 for α-tocotrienol and 410.41 for β/γ-tocotrienol, each of which were radical cations, similarly to what was observed in MS imaging. The β- and γ-tocopherols, and their tocotrienol forms are isomers that could not be differentiated by this analysis. No δ-tocopherols or δ-tocotrienols were observed in the oil or in the imaged embryo sections. A collisional cell energy of 22 was used for fragmentation. Data were plotted as the mol% amounts of the total observed content. Relative amounts of tocochromanols were adjusted using response factors as determined by measuring standards at known concentration.

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Conflict of interest

The authors declare no conflict of interest.

Author Contributions

SS generated, characterized and advanced transgenic plants and performed photosynthesis measurements and yield measurements for greenhouse-grown plants. Field increase was coordinated by TW and KH, total harvested seed cotton was ginned and devitalized by TW. Seed oil was solvent extracted, refined and bleached by MD. Mass spectrometry imaging and mass spectrometry analysis was performed by TR. Vitamin E analyses by HPLC were performed on devitalized seeds or oil samples by AK and WZ under the supervision of EC. EC provided the Napin: HGGT binary vector and provided ongoing advice during the course of the project. SS conducted antioxidant assays with assistance of TR. SS coordinated oxidative stability testing of refined oils. KC and SS coordinated the overall project and developed the first draft of the manuscript. MD, AK, TR, EC assisted with edits to the manuscript. All authors approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 pKan-NapinHvHGGT (16 125 bp) binary vector for Agrobacterium-mediated cotton transformation and direct seed-specific expression of HvHGGT enzyme leading to the production of tocotrienols in cotton embryos.

Figure S2 Flow chart showing steps involved in the development of Cotton Somatic Embryogenic Cell Lines (SECL) and Agrobacterium-mediated cotton transformation [modified from Leelavathi *et al.,* 2004, Rathore *et al.,* 2006, 2015]

Figure S3 (A) PCR confirmation of HGGT T0 and T1 transgenic plants

Figure S4 Tocopherol and Tocotrienol content measured by HPLC showing distinct peaks of tocopherols (α, β/γ and δ) in both Coker 312 (A) and HGGT transgenic lines (B)

Figure S5 MS imaging of relative TAG content and distribution in cotton seed/embryo sections

Figure S6 Mol per cent composition of triacylglycerol molecular species and tocochromanols from refined bleached cottonseed oil

Figure S7 MS and MS/MS of tocochromanols observed in refined bleached cottonseed oil

