Full Length Research Paper

Overexpression of homogentisate phytyltransferase in lettuce results in increased content of vitamin E

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Vitamin E is a group of lipid soluble compounds, which are important antioxidants and play a crucial role in mammals and plants. They can protect the membrane from photooxidation, and they are involved in signal transduction and transcription regulation as well. The enzyme homogentisate phytyltransferase (HPT) has been demonstrated to be a key enzyme limiting tocopherol biosynthesis. In this study, *HPT* gene isolated from *Lactuca sativa* L. (*LsHPT*) was transferred into *L. sativa* (lettuce) via *Agrobacterium*-mediated method and 15 transgenic plants were obtained. The expression of *LsHPT* gene and the total content of α and γ-tocopherol in transgenic plants were increased up to 4 and 2.61 folds, respectively, demonstrating that overexpression of *HPT* gene is an effective way to improve vitamin E content in lettuce.

Key words: *Lactuca sativa*, tocopherol, homogentisate phytyltransferase (HPT), *Agrobacterium*-mediated transformation, real-time fluorescent quantitative-polymerase chain reaction (qRT-PCR).

INTRODUCTION

Vitamin E is a group of lipid soluble compounds, which are termed tocochromanols. It was first recognized in 1922 and found to be an important dietary factor for animal reproduction (Evans and Bishop, 1922). More than 40 years passed before the vitamin E was associated with an antioxidant property (Epstein et al., 1966). During the last 10 to 20 years, the roles of tocochromanols in human and other mammals were studied extensively. Tocochromanols are required for maintaining proper muscular, immune and neural functions. Epidemiological evidence indicates that increased dietary intake of vitamin E can result in decreased risk of certain cancers, cardiovascular diseases, cataracts and age-related decline of the immune system (Brigelius-Flohe et al., 2002). In mammals, tocochromanols can have contact with the membrane molecules and scavenge and quench various reactive oxygen species (ROS) and protect the membrane from photooxidation (Brigelius-Flohe and Traber, 1999). Tocochromanols are reported to be involved in several signal transduction pathways and transcription regulation in mammals as well (Azzi et al., 2004). The function of tocochromanols in plants was firstly thought to be associated with its antioxidant activity in the maintenance of membrane integrity (Fryer, 1992). Recent evidence suggested that tocochromanols play a number of functions in plants (DellaPenna and Pogson, 2006), showing that they may play similar roles in mammals (Munné-Bosch, 2005).

There are eight types of tocochromanols, the consistent structure of which contains a hydrophilic chromanol head group and a hydrophobic prenyl tail. The head group is derived from homogentisic acid (HGA), while the tail is derived either from phytyl diphosphate (PDP) (Rohmer, 2003) or geranylgeranyl diphosphate (GGDP) (Cahoon et al., 2003). Based on the different origins of the tail group,

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Abbreviations: DRI, Dietary reference intake; **FW,** fresh weight; **GGDP,** geranylgeranyl diphosphate; **HGA,** homogentisic acid; **HPLC,** high performance liquid chromatography; **HPT,** homogentisate phytyltransferase; **MPBQ,** 2-methyl-6-phytyl-1,4-benzoquinol; **PDP,** phytyl diphosphate; **PFD,** photon flux density; **RACE,** rapid amplification of cDNA ends; **ROS,** reactive oxygen species.

tocochromanols can be separated into two groups, tocopherols and tocotrienols. The former has a PDP-derived tail and the latter has a tail that originates from GGDP. According to the position and number of the methyl substituents, both tocopherols and tocotrienols can be further divided into four types: α, β, γ and δ types. Tocopherols, especially α-tocopherol has been revealed to have the most important role in vitamin E activity among all the tocochromanols (Eitenmiller, 1997; Schneider, 2005; Weiser et al., 1996). Moreover, it is reported that only α-tocopherol is retained and distributed throughout the body during digestion (Shintani and DellaPenna, 1998).

Tocochromanols have been found in photosynthetic bacteria, fungi, algae, plants and animals (Grusak and DellaPenna, 1999). However, they can only be synthesized by photosynthetic organisms, including higher plants and cyanobacteria (Sattler et al., 2003). Apart from the bypass pathway catalyzed by phytol kinase, there are at least five steps in the biosynthetic pathway of tocochromanols. The following enzymes are involved in the pathway: 4-hydroxyphenylpyruvate dioxygenase, homogentisate phytyltransferase, homogentisate geranylgeranyl transferase, MPBQ methyltransferase, tocopherol cyclase and γ-tocopherol methyltransferase. The enzyme homogentisate phytyltransferase (HPT) catalyzes the condensation of HGA with PDP yielding 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ). The enzyme HPT was primarily isolated from *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana* L. (Collakova and DellaPenna, 2001; Savidge et al., 2002; Schledz et al., 2001). It has been demonstrated that HPT is a key enzyme limiting tocopherol biosynthesis both in unstressed and abiotic stressed *Arabidopsis* leaves (Collakova and DellaPenna, 2003a, b). It was reported that overexpression of HPT in Arabidopsis led to increase of tocopherol content by up to 4.4-fold and 75% in leaf and seed, respectively (Collakova and DellaPenna, 2003a; Savidge et al., 2002).

Lactuca sativa (lettuce) is a leafy vegetable which is commonly used in various dishes and can be eaten raw as well. It is rich in β-carotene, and vitamins E, C and B. However, the vitamin content, especially the vitamin E in lettuce is still below the dietary reference intake (DRI). Thus, increasing vitamin E content in *L. sativa* can be quite meaningful. Genetic engineering of vitamin E biosynthesis pathway may be a valuable candidate strategy for increasing α-tocopherol content in lettuce. In our former work, the enzyme HPT, designated as LsHPT, was isolated from *L. sativa*. Transient expression of *LsHPT* in *L. sativa* resulted in increased α-tocopherol content in leaves (Ren et al., 2011). The results suggested that the enzyme LsHPT might be a rate-limiting enzyme in tocopherol biosynthesis in lettuce. In this study, *LsHPT* was transferred into *L. sativa* via *Agrobacterium*-mediated transformation, and the gene expression level and the tocopherol content of the transgenic lines were detected.

MATERIALS AND METHODS

Tissue culture conditions

L. sativa L. var. *longifolia* Lam. and *Agrobacterium tumefaciens* strain EHA105 were kept in our laboratory. The pCAMBIA2300 vector was purchased from Cambia Company (Australia) and modified in our laboratory.

Seedlings and tissues were cultivated at controlled temperature (22/18°C, day/night air temperature) under a 16/8-h (light/dark) photoperiod with a photon flux density (PFD) of 100 to 200 µmol photon/m^2/s (cool white fluorescent tubes). All the plant media were adjusted with 1 M NaOH to pH 5.7, solidified with 2.6 g/L phytagel (Sigma, USA) and autoclaved at 121°C for 20 min.

Vector construction and *Agrobacterium* transformation

The gene encoding the enzyme homogentisate phytyltransferase (HPT), designated as *LsHPT*, was isolated from *L. sativa* in our former work and the GenBank accession number of the full-length cDNA was FJ194492. The coding sequence of *LsHPT* was cloned and constructed into the modified plant expression vector pCAMBIA2300 to create a pCAMBIA2300-35S::LsHPT::NOS construct (35S, the cauliflower mosaic virus (CaMV) 35S promoter; NOS, the nopaline synthethase terminator). This construct was confirmed by sequencing, and was transformed into *A. tumefaciens* strain EHA105 with the freeze-thaw method. The empty pCAMBIA2300 vector was transformed into EHA105 and served as control.

Transformation of lettuce leaf explants

The *Agrobacterium*-mediated genetic transformation of lettuce was carried out with cotyledon discs (McCabe et al., 2001). Lettuce seeds were sterilized in 75% ethanol (1 min) and 10% sodium hypochlorite (10 min), washed in sterile water for five to six times, and sowed on MS_0 solid medium containing 4.4 g/L Murashige and Skoog basal medium (MS) (Sigma, USA) and 30 g/L sucrose. Both ends of each cotyledon from four to seven-day-old seedlings were cut off. *A. tumefaciens* harboring the plant binary expression vector was confirmed by PCR and cultivated at 28°C in YEB medium containing 100 mg/L streptomycin, 50 mg/L rifampicin and 100 mg/L kanamycin until the optical density at 600 nm (OD_{600}) reached 0.8 to 1.0. The culture was recovered by centrifugation at 5,000 rpm for 8 to 10 min, then resuspended in 1/2 MS_0 liquid medium (2.2 g/L MS + 30 g/L sucrose), and the final OD_{600} was 0.5 to 0.6. The *Agrobacterium* suspension was added with 100 µM acetosyringone, and the cotyledons were inoculated with the suspension for 8 to 10 min. The inoculated explants were then transferred to the co-cultivation medium; MS_1 solid medium [4.4 g/L MS + 30 g/L sucrose + 0.5 mg/L 6-benzylaminopurine (6-BA) + 0.1 mg/L 1-naphthaleneacetic acid (NAA)]. After 36 to 48 h co-cultivation in the dark, the explants were transferred and placed onto the selection and regeneration medium; MS_2 solid medium (4.4 g/L MS + 30 g/L sucrose + 0.5 mg/L 6-BA + 0.1 mg/L NAA + 40 mg/L kanamycin + 250 mg/L carbenicillin). The cultivation conditions were described earlier. Every ten days, the leaf discs were transferred onto fresh MS_2 solid medium. After three to four weeks, the adventitious shoots formed from the calli were transferred to MS_3 solid medium (4.4 g/L MS + 30 g/L sucrose + 0.046 mg/L 6-BA + 0.026 mg/L NAA + 40 mg/L kanamycin + 250 mg/L carbenicillin) after removal of the surrounding calli for shoot growth. After two weeks, they were transferred to the rooting medium; MS_4 solid medium (4.4 g/L MS + 30 g/L sucrose + 20 mg/L kanamycin + 250 mg/L carbenicillin) for root induction. Afterward, the medium was washed away and the plantlets were transferred into perlite for

acclimation. After two weeks, the plantlets were transplanted into pots filled with a mixture of peat, vermiculite and perlite (peat : vermiculite : perlite = 6:3:1). *A. tumefaciens* harboring an empty pCAMBIA2300 vector were also adopted in the lettuce transformation as the control.

PCR analysis of kanamycin resistant lettuce plants

DNA was extracted from these kanamycin resistant plantlets by CTAB based method (Ausubel et al., 2002). The DNA samples were used as the templates of polymerase chain reaction (PCR) which was performed in a mixture (final volume of 25 µl) filled with 1 ′ PCR buffer (Mg^{2+} plus), 1.5 unit of Taq DNA polymerase (TaKaRa, Japan), 2 µl dNTP mixture (0.2 mM each) and 0.4 µM of each primer (35SF (5'-TTCGTCAACATGGTGGAGCA-3') and HPTR (5'-gGCCATGAACCAACAATCCATCCAAGAG-3'). The PCR was performed by denaturing at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 45 s, 55°C for 50 s and 72°C for 1.5 min) and a final extension at 72°C for 10 min. The PCR products were analyzed by ethidium bromide (EB) stained agarose gel electrophoresis. Lettuce plants transformed with an empty pCAMBIA2300 vector were detected with the primers NPTIIF (5'-CGCCAAGCTCTTCAGCAATATC-3') and NPTⅡR (5'-AGCTGTGCTCGACGTTGTCACT-3') under the same condition as described earlier.

RNA isolation and qRT-PCR analysis

Mature leaves were collected from PCR-positive plants and powdered in liquid nitrogen. Total RNA was isolated according to the manufacturer's instructions (Tian'gen Biotech Company, China). RNA samples were stored at -80°C before use. The quality and concentration of RNA samples were checked by EB-stained agarose gel electrophoresis and spectrophotometer analysis.

The real time fluorescent quantitative reverse transcription-PCR (qRT-PCR) was carried out according to SYBR PrimeScript™ RT-PCR kit (TaKaRa, Japan). The fragment from *LsHPT* was amplified with the primers RTF (5'-ACTTGCTGTGTTTCCAA AGCCTGTGATA-3') and RTR (5'-TCTGCTCCAAAGAAAAGG AGATGATGCC-3'). These primers were designed to span an exon-exon boundary to avoid amplification of any contaminating genomic DNA. The PCR was performed at 95°C for 20 s, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 15 s and 72°C for 25 s) and 70 to 95°C for 10 s. Meanwhile, a fragment from the ubiquitin gene (GenBank accession number AY189972) was also amplified paralleling the *LsHPT* fragment amplification using primers UbiF (5'-AAGACCTACACCAAGCCCAA-3') and UbiR (5'-AAGTGAGCCCACACTTACCA-3'). The amplified products were analyzed with the software Opticon Monitor (MJ research Company, USA). The Ct values of qRT-PCR were calculated with this software and the relative transcriptional expression levels of *LsHPT* gene in the transformants were indicated by $2^{-\Delta\Delta Ct}$ values according to Livak and Schmittgen (2001) method.

Detection of tocopherol contents

High performance liquid chromatography (HPLC) analysis was carried out as follows: mature leaves from PCR-positive plants were powdered in liquid nitrogen, the leaf powder was weighed and hexane was added and was then followed by ultrasonically aided solvent extraction. The ratio of the weight (mg) of the powder to the volume (µl) of the solvent was 1:2. The ultrasonically aided solvent extraction lasted for 30 min. The ultrasonic power and frequency were 40 kHz and 400 W, respectively. After centrifugation at a   high

speed, the supernatant was transferred into a clean microfuge tube and dried in a ventilation cabinet. Methanol was then added into the tube according to the original weight of the leaf powder. The ratio of the weight (mg) of the powder to the volume (µl) of the solvent was 1:1. After complete dissolution, the extraction was filtered (0.2-µm acrodisc syringe filter) into an autosampler tube. Separation of tocopherols extracted from the leaf powder was carried out by reverse phase HPLC on a Hitachi HPLC system (Hitachi, Japan) equipped with an L-2130 pump, an L-2200 autosampler, an L-2300 column oven and an L-2455 Diode Array Detector. The analysis was performed on a C18 silica gel HPLC column (YMC Co., Japan); 4.6 x 150 mm (particle size: 5 µm). The HPLC separation was performed with 2% (v/v) water (solvent A) and 98% (v/v) methanol (solvent B). The column was run at 30°C for 20 min at a flow rate of 1 ml/min, and the injection volume was 30 µl. Because the tocochromanol molecules absorbed at 292 nm, the leaf extraction was monitored with the absorbing wavelength of 292 nm. The tocopherol content was analyzed by external standard method and calculated based on standard curves with the software D2000-Elite (Hitachi, Japan).

RESULTS AND DISCUSSION

### Genetic transformation and PCR analysis

To study the function of *LsHPT* in the vitamin E bio-synthesis of *L. sativa*, the recombinant plant expression vector (pCAMBIA2300-35S::LsHPT::NOS) was constructed. The *LsHPT* was driven by the CaMV 35S promoter, which was a very strong constitutive promoter and could result in high levels of gene expression in dicotyledonous plants.

Cotyledon discs of *L. sativa* were inoculated with *A. tumefaciens* harboring the pCAMBIA2300-35S:: LsHPT:: NOS construct. After co-cultivation, the inoculated explants were transferred to MS_2 solid medium for selection and regeneration (Figure 1a). After two weeks, calli began to form at the cut end of the leaf discs (Figure 1b) and gradually differentiated into adventitious shoots in the following cultivation (Figure 1c). The cotyledon discs inoculated with empty *A. tumefaciens* (not harboring an expression vector) were cultivated as the negative control. Cotyledon derived calli were also formed in the negative control. However, they grew relatively slow on the selection medium, became dark brown in color and could not differentiate into adventitious shoots (Figure 1d). The regenerated shoots were transferred to MS_3 solid medium for further growth (Figure 1e). After two weeks, the regenerated shoots grew up to 2 to 4 cm high and they were transferred to MS_4 rooting medium (Figure 1f). When the root system had well developed, the plantlets were transplanted into perlite (Figure 1g) before been cultivated into a mixture of peat, vermiculite and perlite (Figure 1h). Altogether, 33 resistant plantlets were obtained via *Agrobacterium*-mediated transformation.

Transgenic lines were confirmed by PCR analysis of the kanamycin-resistant plantlets (Figure 2). The 1048 bp fragment amplified with the primers 35SF and HPTR spanned the CaMV 35S promoter and   *LsHPT* coding
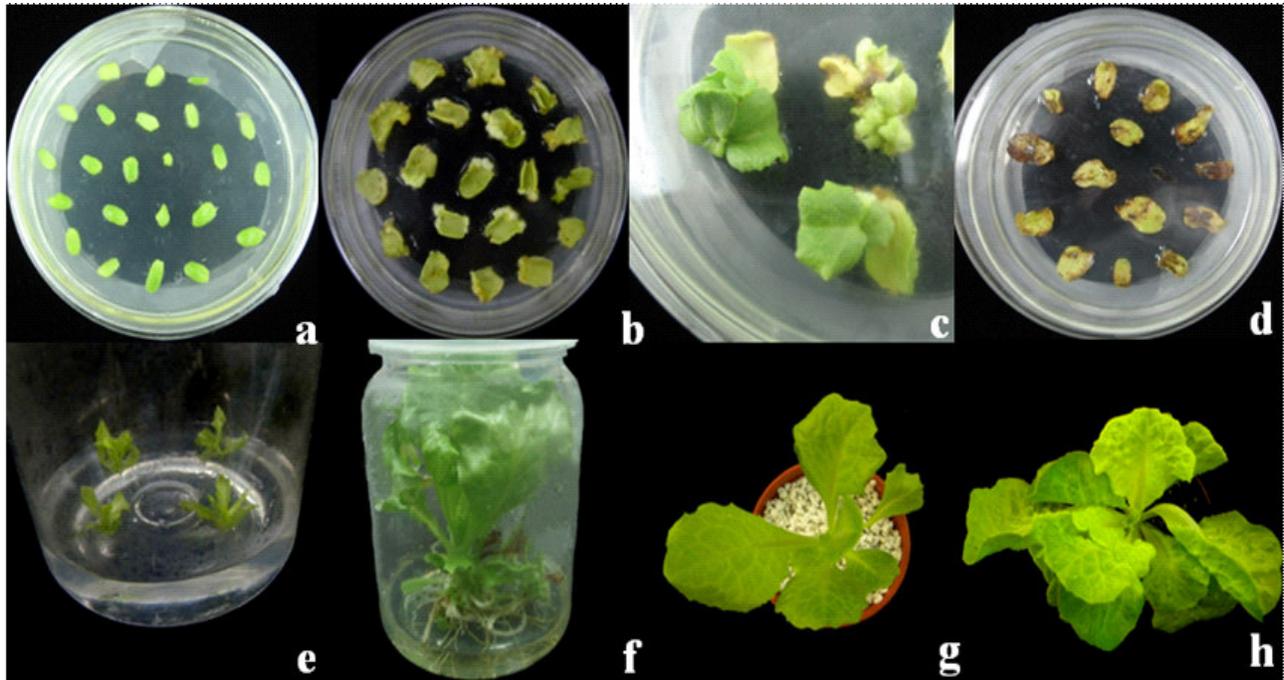
Figure 1. Different stages of *Agrobacterium*-mediated transformation of lettuce. a, Explants after co-cultivation for two days; b, explants cultured on differentiation medium for two weeks after been transformed with EHA105 harboring recombinant vectors; c, regeneration of adventitious shoots; d, explants cultured on differentiation medium for two weeks after been transformed with *A. tumefaciens* strain EHA105; e, culture for stout plantlet; f, taking root; g, domestication of regenerated plant in perlite; h, culture of regenerated plant in soil.
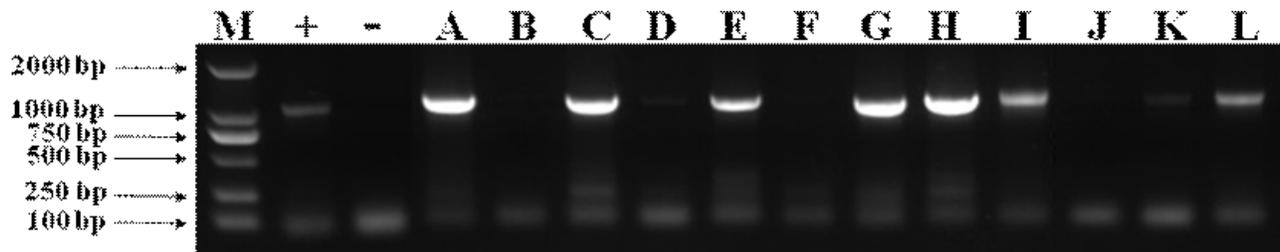

Figure 2. PCR analysis of kanamycin resistant lettuce plants. M, DL 2000 DNA marker; +, positive control; -, non-transformed lettuce; A to L, PCR analysis of *LsHPT* gene in kanamycin resistant lettuce plants.

sequence. *A. tumefaciens* harboring the pCAMBIA2300-35S::LsHPT::NOS construct and genomic DNA extracted from the non-transformed lettuce plants were used as the positive and negative controls, respectively, in the PCR analysis. As a result, 15 plantlets out of the 33 tested were confirmed as transformants via PCR analysis. Plants transformed with *A. tumefaciens* harboring an empty pCAMBIA2300 vector were also confirmed by PCR analysis with the primers NPT II F and NPT II R.

qRT-PCR and HPLC analysis of transgenic plants

The transcriptional level of *LsHPT* gene in transgenic lettuce plants were carried out through real time fluorescent qRT-PCR. A 237 bp fragment from *LsHPT* was amplified with the primers RTF and RTR, while a 196 bp fragment was amplified from the endogenous reference gene (ubiquitin). The relative transcriptional expression levels of *LsHPT* in transformants were indicated by $2^{-\Delta\Delta Ct}$ values. The qRT-PCR results indicate that higher transcriptional levels of *LsHPT* was found in most of the transgenic lines when compared with the negative controls, which were non-transformed plant and plant transformed with *A. tumefaciens*, harboring an empty pCAMBIA2300 vector (Figure 3). Transgenic line 10# presented the highest *LsHPT* mRNA expression level among all the 15 lines, the $2^{-\Delta\Delta Ct}$ value of which were nearly 4 fold when compared with the negative controls,
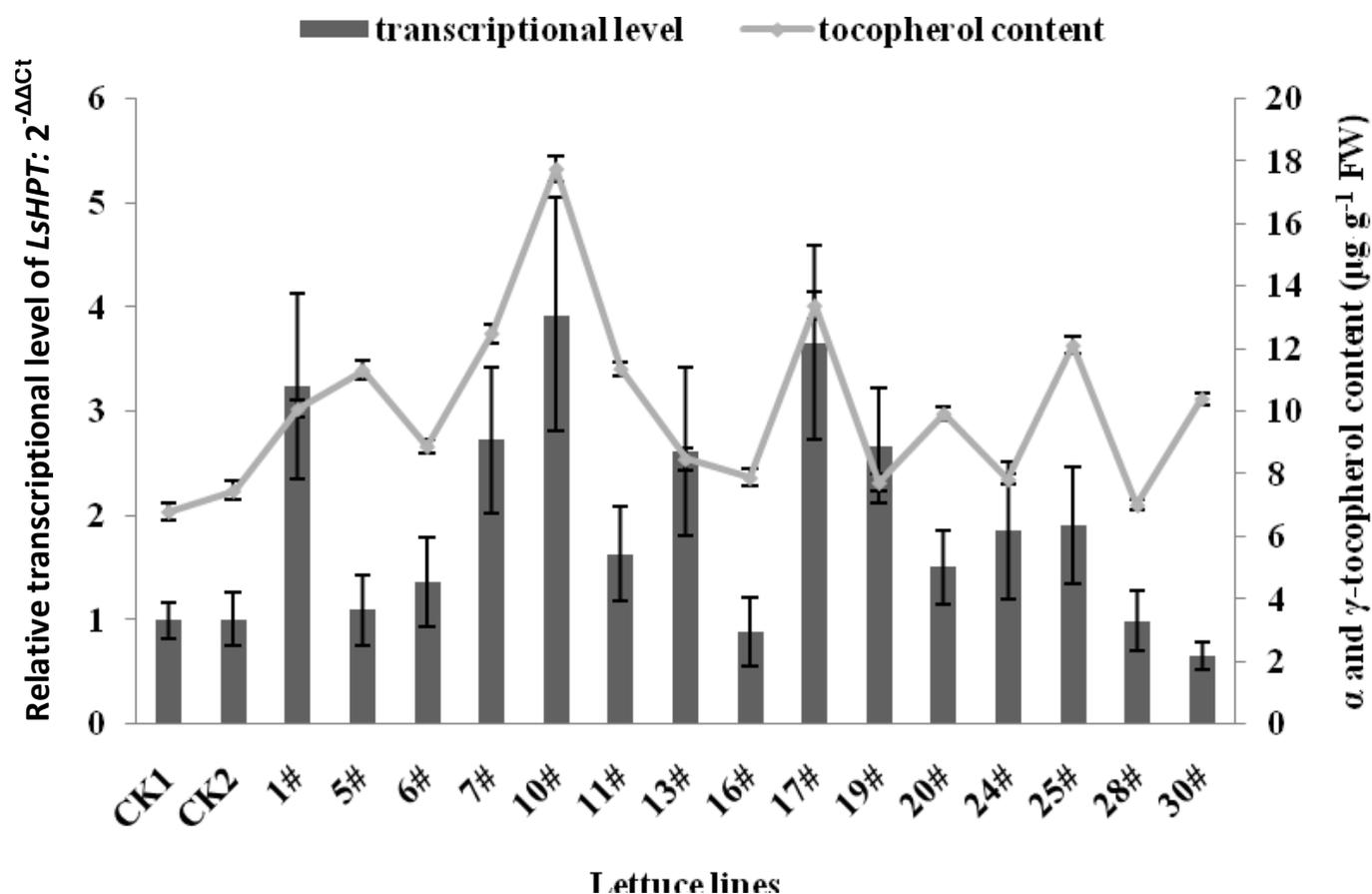
Figure 3. Relative transcriptional level of *LsHPT* gene and α and γ-tocopherol contents in *LsHPT* transgenic lines. CK1, non-transformed plant; CK2, plant transformed with *A. tumefaciens* harboring an empty pCAMBIA2300 vector; 1# to 30#, PCR-positive transgenic plants.

However, transgenic lines 16#, 28# and 30# showed relatively lower transcriptional levels of *LsHPT* when compared with the negative controls. The HPLC results show that the total content of α and γ-tocopherol was increased in most of the transgenic lines. During the biosynthetic pathway of vitamin E, γ-tocopherol is the direct precursor of α-tocopherol. The contents of tocotrienols and other tocopherols were negligible even before or after genetic transformation (data not shown). The total content of α and γ-tocopherol in transgenic lines was increased up to 2.61 fold as compared to that in the non-transformed plant. Transgenic line 10#, which presented the highest *LsHPT* mRNA expression level, also showed the highest tocopherol content [17.77 μg/g fresh weight (FW)] among all the transgenic lines (Figure 3). Neither transcriptional levels nor tocopherol contents showed significant difference between the two negative controls (P > 0.05). The results indicate that the tocopherol content was enhanced and accompanied with the increase of the *LsHPT* mRNA expression level, revealing that *LsHPT* might play an important role in lettuce tocopherol biosynthesis and that it was possible to increase the tocopherol content through genetic engineering.

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