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Engineering tocopherol biosynthetic pathway in lettuce

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Abstract

In order to increase tocopherol content, genes encoding *Arabidopsis* homogentisate phytyltransferase (HPT) and γ-tocopherol methyltransferase (γ-TMT) were constitutively over-expressed in lettuce (*Lactuca sativa* L. var. *logifolia*), alone or in combination. Over-expression of *hpt* could increase total tocopherol content, while over-expression of γ-*tmt* could shift tocopherol composition in favor of α-tocopherol. Transgenic lettuce lines expressing both *hpt* and γ-*tmt* produced significantly higher amount of tocopherol and elevated α-/γ-tocopherol ratio compared with non-transgenic control and transgenic lines harboring a single gene (*hpt* or γ-*tmt*). The best line produced eight times more tocopherol than the non-transgenic control and more than twice than *hpt* single-gene transgenic line.

Additional key words: homogentisate phytyltransferase, *Lactuca sativa,* γ-tocopherol methyltransferase, transformation, vitamin E.

Introduction

Tocopherols are lipid-soluble antioxidants collectively known as vitamin E, synthesized only in plants and other oxygenic, photosynthetic organisms. Tocopherols included four derivatives differing in the number and position of methyl groups on the aromatic ring, named separately as α-, β-, γ-, and δ-form. Among the family, α-tocopherol is believed to have the highest vitamin E activity and naturally synthesized α-tocopherol has advantages than chemically synthesized α-tocopherol (Eitenmiller 1997).

The tocopherol biosynthetic pathway in *Arabidopsis thaliana* was elucidated since 1990s. Generally, the biosynthesis of tocopherol takes place in plastids of higher plants. Two compounds from different metabolic pathways are utilized as precursors, which include homogentisic acid (HGA), derived from shikimate metabolic pathway to form a polar head group and phytyldiphosphate (PDP; Rohmer 2003) from methyl-erythritol phosphate (MEP) pathway for tail synthesis of tocopherols (DellaPenna 2005). There are at least five kinds of enzymes involved in the biosynthetic pathway of tocopherols, excluding the bypass pathway of phytyl-tail synthesis and utilization. HGA is produced from the tyrosine aromatic amino acid catabolite *p*-hydroxy-phenylpyruvate (HPP) by the cytosolic enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD; Norris *et al*. 1995). Condensation of HGA and PDP is catalyzed by homogentisate phytyltransferase (HPT; Collakova and DellaPenna 2001). The product of this reaction, 2-methyl-6-phytylbenzoquinol (MPBQ), is the first phytylquinol intermediate in the pathway and can be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT; Shintani *et al*. 2002). Both MPBQ and DMPBQ are substrates for tocopherol cyclase (TC) to yield the first tocopherols of the pathway, δ-tocopherol and γ-tocopherol, respectively (Porfirova *et al*. 2002). Both δ- and γ-tocopherol can be methylated by γ-tocopherol methyltransferase (γ-TMT) to yield β- and α-tocopherol, respectively (Shintani and DellaPenna 1998).

Increased tocopherols content and α-tocopherol composition in vegetables and crops has been an

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Abbreviations: HGA - homogentisic acid; HPPD - 4-hydroxyphenylpyruvic acid dioxygenase; HPT - homogentisate phytyltransferase; MPBQ MT - 2-methyl-6-phytylplastoquinol methyltransferase; PDP - phytyldiphosphate; TC - tocopherol cyclase; γ-TMT - γ-tocopherol methyltransferase.
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important aim for genetic engineering of vitamin E (Li *et al*. 2008). Significant work has been done for this aim (DellaPenna and Pogson 2006, Guo *et al.* 2009, Seong *et al.* 2009). Previous studies in our laboratory evaluated the contribution of these five enzymes in tocopherol biosynthetic pathway (Li *et al*. 2010). It was found that the γ-*tmt*+*hpt* dual-gene co-overexpression had predominance in improving total tocopherols content (more than 4-fold) and elevating α-tocopherol ratio of tocopherol forms (more than 91 % of total tocopherols) in *Arabidopsis* leaves relative to non-transgenic control (NC). If this result can be applicable in vegetables or crops, it will provide an efficient strategy for development of plants with higher nutrition value.

Lettuce (*Lactuca sativa* L. var. *logifolia*, romanie lettuce) is one of the favorable fresh vegetables consumed worldwide. According to United States Department of Agriculture (USDA) nutrient database the total tocopherol content in lettuce is about 0.5 μg g^{-1}(f.m.), with α-/γ-tocopherol ratio 0.26. Therefore, tocopherol content and composition of lettuce can be improved by the expression of genes involved in the tocopherol biosynthetic pathway.

In previous studies related to tocopherol biosynthetic pathway in lettuce, the cDNA encoding γ-TMT from *Arabidopsis* was over-expressed in lettuce to improve the tocopherol composition (Cho *et al*. 2005). The results from this study indicated that a stable inheritance and expression of *Arabidopsis* γ-*TMT* in lettuce results in the conversion of the γ-tocopherol pool to α-tocopherol in transgenic lettuce. In another study, gene encoding HPT or TC was constitutively over-expressed in lettuce, respectively (Lee *et al*. 2007). Both of the single transgenic lines increased total tocopherol content more than 2-fold, mainly due to an increase in γ-tocopherol. However, there had been no dual-gene overexpression reported yet.

In this study, we report the introduction of gene constructs containing cDNA clones of *hpt* and γ-*tmt* into lettuce, alone or in couple combination, driven by the constitutive cauliflower mosaic virus 35S promoter. Tocopherol content and α-/γ-tocopherol ratio in the leaves of the transgenic lines were investigated.

Materials and methods

Plants and growth conditions: Seeds of wild-type lettuce (*Lactuca sativa* L. var. *logifolia*) were sterilized with 70 % ethanol for 30 s, followed by submersion in *Chlorox* for 10 min. After sterilization, seeds were washed with sterile water and cultured on Murashige and Skoog (1962; MS) medium [4.3 g dm^{-3} MS salts (*Sigma*, St. Louis, USA) + 0.8 % agar + 20 g dm^{-3} sucrose (pH 5.7)]. Seeds were germinated at 25 °C and a 16-h photoperiod (irradiance of 50 μmol m^{-2} s^{-1}).

cDNAs generation and vector construction: Total RNA was isolated from leaves of *Arabidopsis thaliana* (Columbia ecotype) by using *TRIzol* reagent (*Gibco/Brl*, Gaithersburg, MD, USA) and cDNA was generated by using *ReverTra Ace*® (*Toyobo*, Osaka, Japan). The primers were AtHPT-F (5'-ATGGAGTCTCTGCTC TCTAGTTCTTCTC-3') and AtHPT-R (5'-TCACTTCAA AAAAGGTAACAGCAA GTAC-3') for *At-hpt* gene (GenBank accession No. AY089963), AtTMT-F (5'-ATG AAAGCAACTCTA GCAGCACCCTC-3') and AtTMT-R (5'-TTAGAGTGG CTTCTGGCAAGTGATGA-3') for *At-γ-tmt* gene (GenBank accession No. AF104220). A total of 50 μm^3 of reaction solution was pre-denaturated at 94 °C for 2 min, followed by 30 cycles of amplication (98 °C for 10 s, 58 °C for 30 s and 68 °C for 75 s). The 6× myc tag was sub-cloned before the termination codon of the entire coding region as screening marker.

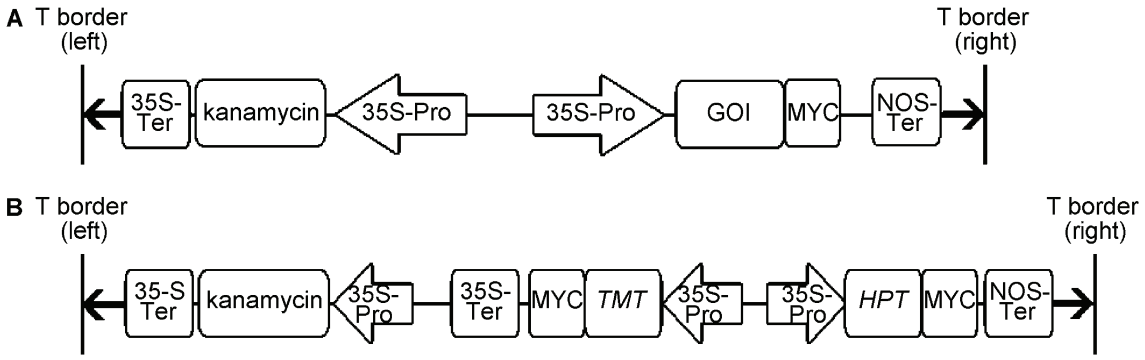

Fig. 1. Schematic representation of the transformation vector constructs used in this study. *A* - schematic representation of the single gene transformation vector construction; *B* - schematic representation of dual expression plasmids construction used in transformation. 35S-Pro - cauliflower mosaic virus 35S promoter; 35S-Ter - cauliflower mosaic virus 35S polyA terminator; Nos-Ter - nopaline synthase gene terminator; GOI - gene of interest; MYC - myc-tag.

Primers AtHPTmyc-F (5'-CCAGATCTATGGAGT CTCTGCTCTCTAGTTC-3', with *Bgl*II digesting site attached), AtTMTmyc-F (5'-CCAGATCTATGAAAG CAACTCTAGCAGCACC-C-3', with *Bgl*II digesting site attached), and myc-R (5'-TTGGTCACCTTACCCCGGG CTGCAGGAATTC-3', with *Bst*EII diegesting site attached) were designed to amplify AtHPT-myc and AtTMT-myc sequences, respectively. The confirmed gene of interest (GOI)-*myc* sequences were digested with restriction enzymes, and inserted into the *Bgl*II-*Bst*EII sites of pCAMBIA2301 vector to construct the single gene transformation vector pCAMBIA2301-*hpt-myc* and pCAMBIA2301-*tmt-myc* (Fig. 1*A*). In order to put two genes in one vector, the expression cassette of vector pBI121 (*Clontech*, CA, USA) was sub-cloned into the *Hin*dIII-*Eco*RI site of pCAMBIA2301 vector to construct the co-expression vector named pCAMBIA2301+. The γ-*tmt-myc* fragment was sub-cloned into *Xba*I-*Sac*I site, while the *hpt-myc* fragment was cloned into *Bgl*II-*Bst*EII site (Fig. 1*B*). The primer AtTMTmyc-F2 (5'-CCT CTAGAATGAAAGCAACTCTAGCAGCACCC-3', with *Xba*I digesting site attached) and myc-R2 (5'-AAG AGCTCTTACCCCGGGCTGCAGGAATTC-3', with *Sac*I digesting site attached) were designed to amplify AtTMT-myc sequence with *Xba*I/*Sac*I digesting sites. All the reading frames of GOI-*myc* sequences were confirmed to be correct by sequencing from both strands.

***Agrobacterium*-mediated transformation of lettuce:** Single colonies of *Agrobacterium tumefaciens* strain EHA105 bearing the pCAMBIA2301::GOI-myc vector were inoculated into 20 cm^3 of yeast extract and beef (YEB) liquid medium containing rifampicin, streptomycin and kanamycin and cultured overnight at 28 °C to A_{600} 1.1 - 1.6. The culture was centrifuged at 4 000 *g* for 15 min and re-suspended in the same volume of liquid MS medium, then diluted to a final volume of 20 times the original culture with MS medium. Cotyledons from 6 to 7-d-old lettuce seedlings were wounded by scratching three times across the abaxial surface perpendicular to the midvein and submerged in 1:20 bacterial-MS for 10 min (Wroblewski *et al*. 2005). They were blot-dried on sterile filter paper, placed on MS1 medium (MS medium supplemented with 0.1 mg dm^{-3} naphthaleneacetic acid, NAA and 0.5 mg dm^{-3} 6-benzylaminopurine, BA) and incubated for 2 d at 25 °C in the dark (Lee *et al.* 2007). The *Agrobacterium*-treated cotyledons were then transferred to MS2 medium (MS1 medium supplemented with 40 mg dm^{-3} kanamycin and 250 mg dm^{-3} carbenicillin). Ten days later they were transferred to fresh MS2 medium. Organogenic shoots from the explants were excised and transferred to MS3 medium (MS medium supplemented with 0.026 mg dm^{-3} NAA, 0.046 mg dm^{-3} 6-BA, 40 mg dm^{-3} kanamycin and 250 mg dm^{-3} carbenicillin). Ten days later, the surviving and strong shoots were planted on MS medium and grown until roots had developed. The rooted plants were transferred and grown in the mixture soil + sand + *Vermiculite* (1:1:1) in greenhouse. Temperature was maintained in the range of 20 to 30 °C during the day and 15 to 25 °C during the night. The irradiance at the canopy level was 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a clear day and photoperiod was 14 to 16 h.

Confirmation of GOI integration: Genomic DNA was isolated from the putative engineered plants and non-transgenic control (NC, transformant with pCAMBIA2301 empty vector) lines using a CTAB method (Ausubel *et al*. 1995). The presence of GOI was detected by PCR to amplify target gene sequence. The primers used for detection were 35S-F (5'-GCACAA TCCCACTATCCTTCGCAA-3', upstream, derived from CaMV35S promoter), TMT-R (5'-GAGTGGCTT CTGGCAAGTGA-3', downstream for *At-γ-tmt*), and HPT-R (5'-CTTCAAAAAAGGTAACAGCAAG-3', downstream for *At-hpt*). In a 200 μm^3 thin-wall PCR tube, the 25 μm^3 reaction mixture contained 10 μM of each PCR primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1.5 U of Taq DNA polymerase (*TaKaRa*, Tokyo, Japan) with 200 ng genomic DNA as template. PCR was carried out on a *Thermo Hybaid* (MA, USA). Amplification procedure for GOI sequence was: 5 min of predenaturation at 94 °C, 32 cycles of amplification (1 min at 94 °C, 1 min at 54 °C, 90 s at 72 °C), followed by 5 min at 72 °C. The amplified products were electrophoresed on 1 % agarose gel.

Confirmation of protein expression of GOI-myc: Protein gel blot analysis was performed as described previously with minor modifications (Yang *et al*. 2000). Fifty μg of total protein, determined by using the *DC* protein assay kit (*Bio-Rad*, Hercules, CA), was fractionated on 12 % SDS-PAGE mini-gel and blotted onto a nitrocellulose membrane (0.45 μm; *Perkin Elmer*, CA, USA). The blots were probed with the primary antibody c-Myc (*9E10*) (*sc-40*, mouse monoclonal IgG1, *Santa Cruz Biotechnology*, CA, USA) [diluted in PBST (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1 % *Tween 20*), washed with PBST three times, reacted with goat anti-mouse IgG-AP (*sc-2047*, *Santa Cruz Biotechnology*), washed, and exposed with alkali phosphatase for 5 min.

Analysis of tocopherol: For each plant, the leaves were harvested. Freeze-dried material (150 mg) was ground in liquid nitrogen, and was extracted with 4 cm^3 of *n*-hexane in dim light and in the presence of argon to prevent the oxidation of vitamin E. After centrifugation at 11 000 *g* for 10 min, the clear supernatant was taken and the pellet was re-extracted twice with 2 cm^3 *n*-hexane. All the resulting supernatants were pooled, evaporated to dryness under nitrogen, dissolved in 750 μm^3 of methanol, and stored at -80 °C until being analyzed. The organic extracts (30 μm^3) were resolved on a *Phenomenex* C18 reverse-phase column (5 μm, 4.6 mm i.d. × 250 mm length; *Calesil ODS-100*, Kyoto, Japan) at 30 °C with a methanol: H_2O (98:2, *v*/*v*) mobile phase for 30 min at the

flow rate of 1 cm^3 min^{-1} prior to rinsing and re-equilibration of the column. Tocopherols were detected by fluorescence with excitation at 292 nm and emission at 325 nm. Tocopherols were identified by retention times and quantified relative to dilution series of commercially available authentic standards (*Sigma*).

Measurement of GOI relative expression level: Total RNA was isolated from leaves and any contaminating genomic DNA was removed by treatment with RQ1-RNase free DNase (*Promega*, Madison, WI, USA). Total RNA (1 µg) was reverse transcribed to generate cDNA in two 20 mm^3 samples using a *Toyobo Rever Tra-Plus* kit according to manufacturer's recommendations. An aliquot of cDNA corresponding to 10 pg to 10 ng of total RNA was used in each real-time PCR assay (*SYBR® ExScript* RT-PCR kit, *TaKaRa*). Partial of polyubiquitin gene was amplified with primers (5'-TCTTAGATC ACCGTCCCATCGT-3' and 5'-TCT GAGATTGTC CGAGGATATGAG-3') as a control (Argyris *et al.* 2008). The SYBR ExScript RT-PCR kit protocol was followed to confirm changes in gene expression. The target messages *ubiquintin*, *At-hpt*, and *At-γ-tmt* mRNA in unknown samples were quantified by measuring the cycle threshold value and extrapolation to calibration graphs constructed with serial dilution templates of known concentrations. The thermal cycle conditions used were 10 s at 95 °C followed by 40 cycles of amplification (5 s at 95 °C and 30 s at 60 °C). Melt curve analysis and agarose gel electrophoresis following each real-time PCR, were performed to assess product specificity. A comparative method for quantification was adopted. The expression level ratios of both *hpt /ubiquintin* and *γ-tmt /ubiquintin* in non-transgenic lettuce were initialized as "1.0", thus the relative expression levels in all samples were determined.

Results and discussion

After *Agrobacterium*-mediated transformation, we transferred callus to selection medium (MS2 medium) containing kanamycin. Green calluses were formed near the sites of wounding of the lettuce explants (Fig. 2*A*). Continuous selection of the proliferating tissues for two weeks resulted in the appearance of vigorously growing green leaves (Fig. 2*B*). This was transferred to regeneration medium. Plants were successfully regenerated and developed roots when transferred to the rooting medium (Fig. 2*C*). The rooted plants were

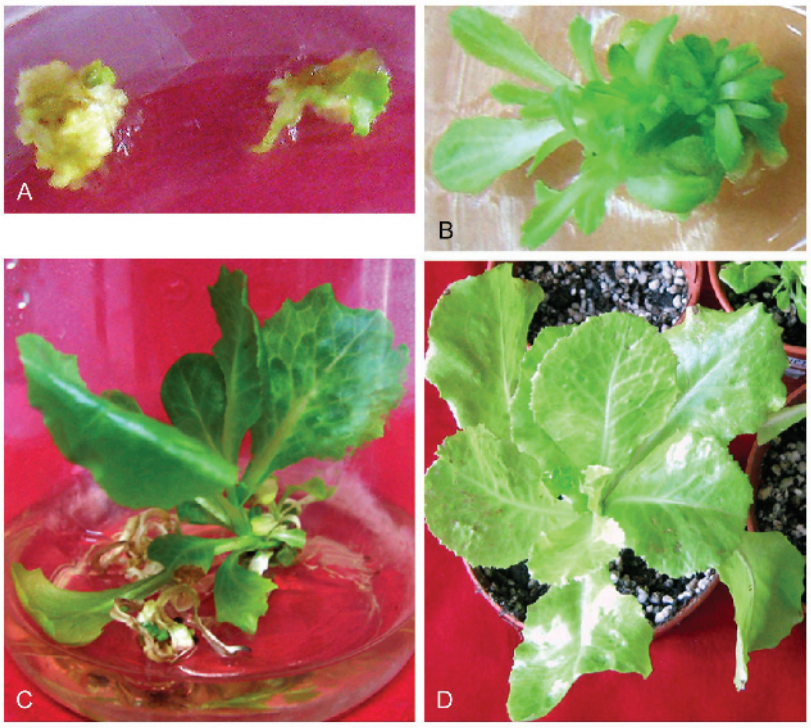

Fig. 2. Generation of transgenic lettuce plants by *Agrobacterium*-mediated transformation: *A* - after *Agrobacterium* co-cultivation, green calli formed near the wounded sites on MS1 medium; *B* - organogenic shoots from the explants were formed on MS2 medium (MS1 medium supplemented with phytohormones, kanamycin and carbenicillin); *C* - roots of regenerated green shoot were developed after being transferred to basal MS medium; *D* - the root-developed plants were transferred and grown in the soil mixture in greenhouse.

Table 1. Gene constructs and derived transgenic lines. The antibiotic resistance gene (*kan*) is always placed near the left border of the T-DNA (a portion of the tumor-inducing plasmid that is transferred to plant cells).

		Number of Kan resistant	Number of PCR-positive	Representative lines
kan-hpt	EHA105	101	22	5, 6, 9, A5, A8, A16, B5, B14
kan-tmt	EHA105	122	24	6, 11, 12, 14, 16, 20, 28, 36
kan-tmt-hpt	EHA105	105	17	1, 4, A11,B2, C8, D16, D18

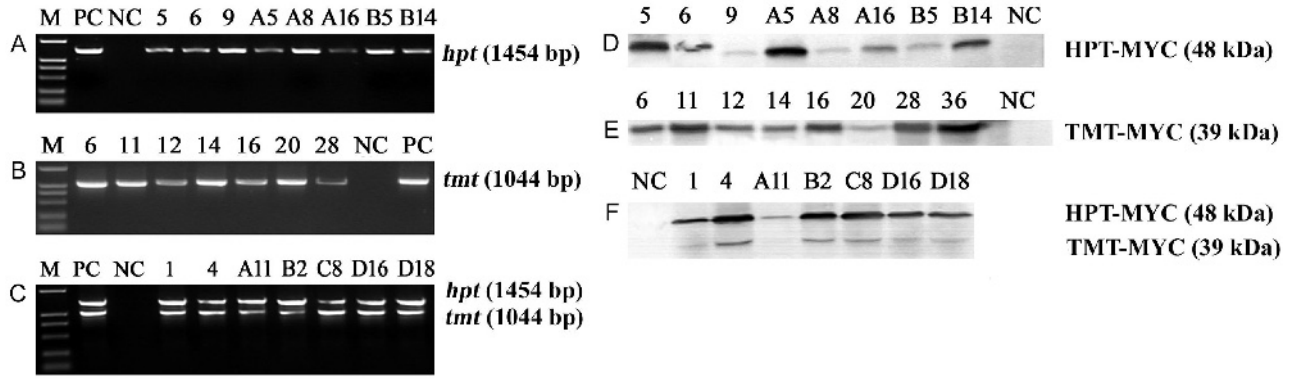

Fig. 3. Molecular analysis of transgenic lines: *A* - representative PCR analyses for the presence of *hpt* gene in *hpt* single overexpression lines; *B* - representative PCR analyses for the presence of *γ-tmt* gene in *γ-tmt* single transgenic lines; *C* - representative PCR analyses for the presence of *hpt* and *γ-tmt* genes in *γ-tmt+hpt* dual overexpression lines (M - DL-2000 marker, PC - positive control, NC- non-transgenic control); *D* - Western blot of *hpt* single transgenic lines; *E* - Western blot of *γ-tmt* single transgenic lines; *F* - Western blot of *γ-tmt+hpt* dual transgenic lines (NC - non-transgenic control).

transferred *ex vitro* and grown in the soil mixture in greenhouse (Fig. 2*D*). We regenerated more than 100 independent lines of *hpt*, *γ-tmt*, and *γ-tmt+hpt*, respectively, of transgenic lettuce plants (Table 1).

Integration of the genes of interest (GOI) into the lettuce genome was confirmed by PCR analysis using forward primer which was derived from CaMV35S promoter and reverse primer which was GOI-specific. 22 of the putative *hpt* independent transformants were found containing 1 454 bp target fragments, and non-transgenic control (NC, transformant with pCAMBIA2301 empty vector) lines showed no specific fragments amplified (Fig. 3*A*). Similarly, 24 of the putative *γ-tmt* independent transformants were found to contain 1 044 bp *γ-tmt* fragments (Fig. 3*B*), and 17 of the putative *γ-tmt+hpt* independent transformants contained both of *hpt* and *γ-tmt* fragments (Fig. 3*C*).

Total proteins were extracted from leaves of PCR-positive lettuce lines, probed with anti-myc antibody, and the proteins of NC lines were also probed at the same time. The transgenic lines expressed target protein fused with myc-tag and the endogenous genes of lettuce could not be probed by specific anti-myc antibody. The *hpt* and γ-*tmt* transgenic lines showed about 48 and 39 kDa immunoblot fragments, respectively (Fig. 3*D*,*E*), while γ-*tmt*+*hpt* dual transgenic lines showed both immunoblot fragments mentioned above (Fig. 3*F*).

Total tocopherol contents in most *hpt* single transgenic lines increased [ranging from 10.81 to 40.41 μg g^{-1}(f.m.)] compared to NC lines [7.10 ± 1.39 μg g^{-1}(f.m.)]. The α-/γ-tocopherol ratio were not significantly changed in *hpt* transgenic lines (ranging from 0.18 to 0.53) compared to NC (about 0.45). In γ-*tmt* single transgenic lines, total tocopherol contents increased only 1.14- to 1.75-folds compared with NC lines, while the α-/γ-tocopherol ratio were greatly improved in these lines, ranging from 3.90 to 9.27. In γ-*tmt*+*hpt* dual transgenic lines, total tocopherol content was increased up to 64.55 ± 2.31 μg g^{-1}(f.m.). Meanwhile, α-/γ-tocopherol ratio was also increased in dual transgenic lines, ranging from 1.32 to 8.56 (Table 2).

Real-time PCR analysis showed a good correlation between mRNA levels and tocopherol content/ composition in single gene transgenic lines. In general, transformants whose *hpt* mRNA levels were higher, had relatively higher tocopherol contents, while elevated *γ-tmt* expression levels might increase α-/γ-tocopherol ratio. However, in dual transgenic lines, there was complicated relationship between GOI expression levels and tocopherol contents (Fig. 4). In dual transgenic line HTC8, which had the highest tocopherol content, *hpt* expression level was not the highest one in all these lines. In reverse, its *γ-tmt* expression level was the highest. It seemed that high tocopherol contents were associated

Table 2. Tocopherol content [μg g^{-1}(f.m.)] and α-/γ-tocopherol ratio in leaves of single and double T_0 transgenic plants and non-transgenic control (NC). H - *hpt* transgenic lines, T - *γ-tmt* transgenic lines, HT - *hpt* and *γ-tmt* dual transgenic lines. Means ± SE, *n* = 3.

Line	Content	α-/γ- ratio	Line	Content	α-/γ- ratio	Line	Content	α-/γ- ratio
H-2	14.62 ± 1.79	0.51	T-3	10.07 ± 3.14	5.56	T-74	10.39 ± 1.31	5.97
H-5	33.23 ± 2.11	0.49	T-6	10.62 ± 2.91	6.79	T-83	10.17 ± 1.49	4.84
H-6	15.35 ± 1.29	0.19	T-11	9.22 ± 1.32	5.03			
H-9	11.74 ± 1.94	0.30	T-12	10.26 ± 1.05	6.66	HT-1	12.04 ± 1.36	1.32
H-11	18.46 ± 1.31	0.34	T-14	8.72 ± 1.09	4.95	HT-4	22.87 ± 2.31	2.27
H-14	29.72 ± 1.87	0.38	T-16	12.35 ± 2.53	9.03	HT-7	51.49 ± 1.36	5.92
H-18	32.46 ± 1.56	0.42	T-19	9.45 ± 1.87	5.38	HT-22	49.25 ± 2.87	5.38
H-25	17.28 ± 0.94	0.44	T-20	8.87 ± 0.97	4.81	HT-A5	53.87 ± 1.94	6.41
H-31	25.87 ± 1.79	0.50	T-28	10.42 ± 0.80	6.88	HT-A11	56.60 ± 2.81	7.09
H-A4	30.07 ± 1.56	0.25	T-33	11.79 ± 2.41	9.27	HT-A14	44.87 ± 1.11	6.16
H-A5	37.78 ± 0.97	0.37	T-36	9.98 ± 1.16	5.96	HT-B2	37.02 ± 1.93	5.70
H-A8	28.33 ± 2.98	0.40	T-45	8.92 ± 2.38	5.01	HT-B9	36.29 ± 1.36	4.98
H-A9	27.84 ± 1.45	0.34	T-46	11.34 ± 1.94	6.31	HT-C8	64.55 ± 2.31	8.34
H-A16	15.71 ± 1.34	0.18	T-47	12.44 ± 2.01	9.19	HT-C17	28.65 ± 1.38	1.68
H-A21	21.12 ± 3.07	0.45	T-52	10.09 ± 1.34	5.89	HT-D4	19.68 ± 2.77	1.54
H-B5	40.41 ± 1.92	0.46	T-54	9.68 ± 1.41	5.16	HT-D6	58.36 ± 2.83	8.56
H-B11	19.89 ± 1.27	0.40	T-59	9.25 ± 0.78	4.60	HT-D13	50.38 ± 2.76	3.31
H-B14	10.81 ± 2.24	0.48	T-61	8.94 ± 1.65	4.24	HT-D15	60.82 ± 1.94	7.67
H-B15	22.44 ± 0.97	0.47	T-66	10.87 ± 1.33	6.47	HT-D16	42.24 ± 1.25	6.14
H-B17	34.98 ± 1.37	0.48	T-69	9.66 ± 0.94	5.15	HT-D18	20.04 ± 1.91	1.96
H-C3	26.69 ± 0.88	0.53	T-70	9.45 ± 1.28	5.09			
H-C10	29.46 ± 1.98	0.38	T-71	8.07 ± 0.62	3.90	NC	7.10 ± 1.39	0.45

with a coordinative effect of both *hpt* and *γ-tmt* expression levels, and similar phenomena were observed in the dual transgenic line HTD16 and HT4.

Seeds of T_0 transgenic plants were obtained by self-pollination. T_1 progenies of dual transgenic lines were analyzed in order to prove whether there was stable inheritance. Take the dual transgenic lines HT4 for example, 28 progenies were detected to have GOI insertion in genome by PCR among 37 T_1 progenies. Total tocopherol content of these 28 progenies was ranging from 18.41 to 55.23 μg g^{-1}(f.m.), and the average content was (35.33 ± 8.14) μg g^{-1}(f.m.), which was 5-fold higher than in NC lines. The total tocopherol content in T_0

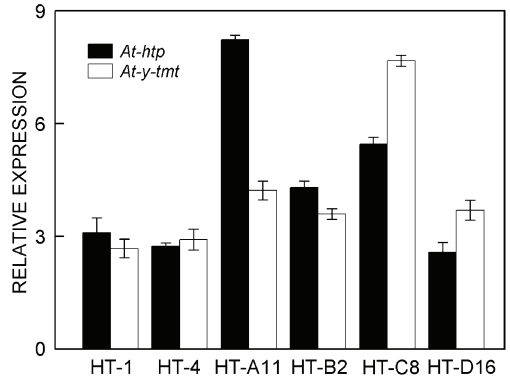

Fig. 4. Real-time analyses for the relative expression level of *hpt* and *γ-tmt* in dual transgenic lettuce lines.

Table 3. Tocopherol content [μg g^{-1}(f.m.)] and α-/γ-tocopherol ratio in leaves of T_0, T_1 and T_2 generation plants in dual transgenic line 4. NC - non-transgenic control. Means ± SE, *n* = 3.

Generation	Lines	Content	α-/γ- ratio
T_0	NC	7.10 ± 1.39	0.45
T_1	4	22.87 ± 2.31	2.27
T_2	4-1	29.82 ± 2.49	2.33
	4-1-1	42.67 ± 2.20	6.01
	4-1-2	16.97 ± 2.43	1.53
	4-1-3	39.44 ± 3.86	5.55
	4-1-4	36.66 ± 3.15	5.16
	4-1-5	30.53 ± 1.83	4.30
	4-1-6	27.48 ± 1.41	3.89
	4-1-7	24.45 ± 1.59	2.44
	4-1-8	31.72 ± 3.29	4.46
	4-1-9	31.25 ± 3.64	4.40
	4-1-10	34.46 ± 2.46	4.85

generation of the same line was 22.87 ± 2.31 μg g^{-1}(f.m.), and it seemed that tocopherol content in T_1 progenies was mostly higher than in T_0 generation. The α-/γ-tocopherol ratio of 28 progenies was ranging from 0.83 to 3.73, and the average was 2.07 ± 0.66. It was similar with the T_0 generation (α-/γ- ratio was 2.27).

T_1 generation of dual transgenic lines HT1 and HTC8

were also detected to have PCR-positive progenies, which had both tocopherol content and α-/γ- ratio increasing. The transgenic line HTC8, which had the highest content of total tocopherol in T_0 generation [64.55 μg g^{-1}(f.m.)], also maintained high level of tocopherol content, ranging from 38.10 to 59.16 μg g^{-1}(f.m.). The T_1 progenies of transgenic line HT1 showed increased total tocopherol content [ranging from 16.97 to 42.67 μg g^{-1}(f.m.)] compared with its T_0 generation. The tocopherol contents of the two transgenic lines were elevated up to 5.27- and 3.33-fold than NC lines for the best-performing events, respectively. The α-/γ-tocopherol ratio of HT1 and HTC8 lines were increased up to 3.45 and 9.73 for the best-performing events, respectively. It was confirmed that T_1 progenies could maintain the elevated tocopherol levels and improved tocopherol composition.

Progenies of a selected transgene-homozygous T_1 plants HT4-1 were used to examine transgenic stability in the subsequent generations by determining the tocopherol contents of ten T_2 plants (Table 3). Except for the line HT4-1-2, all the T_2 generation plants had higher tocoperol contents than the T_0 generation. Seven T_2 generation plants had higher tocopherol contents than the T_1 generation, while other low-content plants (HT4-1-6 and HT4-1-7) did not show significantly decreasing. Considering tocopherol composition, all the T_2 generation plants had higher α-/γ-tocopherol ratio compared with both T_0 and T_1 generations. Specially, the α-/γ-tocopherol ratio was consisting with the tocopherol contents, giving hints on the γ-TMT function.

In conclusion, we reported a strategy to improve tocopherol content and composition in *Lactuca sativa* by genetic manipulation. The genes of two enzymes (HPT and γ-TMT) from *Arabidopsis*, were cloned and integrated into the lettuce genome, alone or in combination. Similarly with the previous study in *Arabidopsis*, dual expression of *γ-tmt+hpt* could increase the total tocopherol content and enhance α-tocopherol proportion at the same time. The total tocopherol content was higher in *γ-tmt+hpt* dual transgenic lines than in *hpt* or *γ-tmt* single transgenic lines. It seems that the γ-TMT plays a more important role in stimulating á-tocopherol accumulation whereas the overexpressed HPT only increased total tocopherol content. Compared with *Arabidopsis*, nine-fold increase of total tocopherol content was observed in dual transgenic lines. Further study on T_1 and T_2 progenies of dual transgenic lines should confirm stable inheritance in tocopherol content and composition.

Over-expressing multiple biosynthetic pathway genes will have cumulative functions in increasing content and composition of target product. Transgenic plants harboring two or more genes of one or more metabolic pathways may be more efficient than transgenic plants harboring only one gene. This result provides an efficient and feasible strategy for genetic engineering of vegetables and crops with higher nutrition value.

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