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BRIEF COMMUNICATION

Overexpression of homogentisate phytyltransferase (*HPT*) and tocopherol cyclase (*TC*) enhances α-tocopherol content in transgenic tobacco

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Abstract

Photosynthetic organisms synthesize the amphipathic antioxidants called tocopherols which are essential components of the human diet. To increase the α-tocopherol (vitamin E) content, *Arabidopsis* genes encoding homogentisate phytyltransferase (*HPT*) and tocopherol cyclase (*TC*) were constitutively expressed individually and in combination (*HPT:TC*) in tobacco plant by *Agrobacterium* mediated transformation. The transgene was confirmed by polymerase chain reaction (PCR), transgene expression was studied by reverse transcriptase (RT)-PCR, integration of the transgene in the plant genome was confirmed by Southern blot, and α-tocopherol content was quantified using high performance liquid chromatography (HPLC). The α-tocopherol content in transgenic tobacco plants expressing *HPT, TC,* and *HPT:TC* was increased by 5.4-, 4.0-, and 7.1-fold, respectively, when compared to the wild type (WT). These results indicate that, the HPT and TC activities are critical for enhancing the vitamin E content in tobacco plants.

Additional key words: *Agrobacterium* mediated transformation, RT-PCR, Southern blot.

Tocopherols not only perform vital functions in plant cells but are also essential or beneficial to humans (collectively known as vitamin E). They protect plant cells against oxidative and photo-oxidative stresses and damages (Munne-Bosch and Alegre 2002, Munne-Bosch and Falk 2003), *e.g.*, by protection of polyunsaturated fatty acids from lipid peroxidation in the chloroplast membrane (Hugly and Somerville 1992). Recently, there is a great interest in manipulating the tocopherol biosynthetic pathway in plants to convert the less active tocopherols to the most bioactive α-tocopherol which has more significant health benefits. The vegetable crops can be an ideal target for the improvement of tocopherol composition.

The manipulation of the metabolic pathway by genetic engineering is often successful if the regulation of the respective pathway is well characterized. The identification of the genes involved in the respective biosynthetic pathway permits new insights into its regulation. From the literature, it is clear that two key enzymes in tocopherol biosynthesis are homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC) (Collakova and DellaPenna 2003a,b, Kanwischer *et al.* 2005). Therefore, we aimed to increase the α-tocopherol content by overexpressing the genes encoding homogentisate phytyltransferase (*HPT*) and tocopherol cyclase (*TC*) individually and in combination using a model plant tobacco. We have also investigated the antioxidant capacities in tobacco leaves of both wild and transgenic plants.

The full length *Arabidopsis HPT* cDNA clone (1.2 kb) (Resource number: PDA 05721 and GenBank acc. number AT2G18950) was procured from *RIKEN*, Ibaraki, Japan. The *HPT* cDNA was cloned in *pDrive*

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Abbreviations: BAP - benzylaminopurine; CTAB - cetyltrimethylammonium bromide; DPPH - 1,1-diphenyl-2-picrylhydrazyl; FRAP - ferric reducing antioxidant power; HPLC - high performance liquid chromatography; HPT - homogentisate phytyltransferase; IAA - indoleacetic acid; MS - Murashige and Skoog; RT-PCR - reverse transcriptase - polymerase chain reaction; TC - tocopherol cyclase; WT - wild type.
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vector (*Qiagen*, Hilden, Germany) and subcloned into *pNutKan* binary vector and finally the construct was transformed to *Agrobacterium tumefaciens* EHA105 by freeze-thaw method (Hofgen and Willmitzer 1988). The *TC* gene construct in pBinAR vector was a gift from Dr. Peter Dormann, Max-Planck-Institute for Molecular Plant Physiology, Potsdam-Golm, Germany, and it was directly transformed to *Agrobacterium tumefaciens* EHA105. The *Agrobacterium* harbouring these two constructs (*HPT* and *TC*) were used for tobacco genetic transformation.

Pre-cultured tobacco (*Nicotiana tabacum* L. var. *xanthi*) leaves on Murashige and Skoog (MS) medium with 0.1 mg dm^{-3} indole-3-acetic acid (IAA) + 1 mg dm^{-3} 6-benzylaminopurine (BAP) were used for *Agrobacterium* mediated genetic transformation. The explants were infected with *Agrobacterium* harbouring *HPT* and *TC,* respectively, for 10 min, blotted dry in sterile filter paper (10 min), explants were transferred to co-cultivation medium (MS + 0.5 mg dm^{-3} IAA + 2 mg dm^{-3} BAP), and incubated in dark for 3 d. After co-cultivation, the explants were transferred to selection medium with 0.1 mg dm^{-3} IAA + 1 mg dm^{-3} BAP + 100 mg dm^{-3} kanamycin + 300 mg dm^{-3} carbenicillin for callus induction and plant regeneration. The explants were subcultured in the same medium once after 25 d. Later, the putative transformants were transferred to half-strength MS medium with 100 mg dm^{-3} kanamycin + 300 mg dm^{-3} carbenicillin for root induction. After the induction of shoots and roots, the plants were transferred to coir pith for acclimatization and finally to soil.

DNA was isolated from leaves of putative transgenic lines by cetyltrimethylammonium bromide (CTAB) method and the DNA yield was measured using a nano-photometer (*Implen*, Munich, Germany) and absorbance was read at 260 nm. The putative *HPT* and *TC* transformants were confirmed for the presence of transgene by PCR using HPT and TC specific primers: *HPT* forward - CGCGTGGTACCTCGAGTGAGTCTC TGCTCTCTAGTTCTTC, *HPT* reverse - CGTCGTGCG GCCGCCTTCAAAAAAGGTAAC, *TC* forward - AGC TGGTACCTATGGAGATACGGAGCTTGATTGTTT, and *TC* reverse - GACTTCTAGAGTTACAGACCCGG TGGCTTGAAGAAA.

Total RNA was isolated from leaves (100 mg) of WT, and *HPT*, *TC*, and *HPT*:*TC* transgenic lines by using *Tri Reagent*® (*Ambion*, Austin, TX, USA). The RT-PCR was performed as per the instruction given in one step RT-PCR kit (*Qiagen*). PCR was performed with HPT and TC specific primers, and to check the integrity of the cDNA and normalize the amount of cDNA template in each reaction, a corresponding 26S rRNA (internal reference standard) was amplified with specific primers: 26S forward - CACAATGATAGGAAGAGCCGAC and 26S reverse - CAAGGGAACGGGCTTGGCAGAATC.

The T_0 tobacco plants were analyzed by Southern blot hybridization for the integration of *HPT* and *TC* genes, respectively. Total genomic DNA was extracted from young leaves of PCR confirmed plants. Plant genomic DNA (5 μg) was digested with *Pst* I and *Not* I for *HPT* (1.2 kb) and *Hind* III and *Eco*R1 restriction enzymes for *TC* (2 kb) at 37 °C for 5 h, separated in 0.8 % (m/v) agarose gel and blotted on positively charged nitrocellulose membrane (*BioTrace NT*, Pall, USA). Hybridization and detection was done with biotin labelling and detection kit (*Fermentas*, Amherst, NY, USA). The labelling probes, hybridization and washing the membrane were performed according to the protocol of *Fermentas* detection system. The biotin labelled *HPT* and *TC* gene PCR products were used as a probe.

The leaves of the transgenic plants (1 g) were grounded with 1 cm^3 of methanol and used for the HPLC (*Shimadzu*, Kyoto, Japan). HPLC system with the photodiode array detector (PDA) was used for the quantification of α-tocopherol. The column, *Phenomenex C*18 5 m (250 × 4.6 mm), was maintained at 24 °C. Methanol (HPLC grade) was used as the mobile phase for the separation. The flow rate was 0.5 cm^3 min^{-1}, the sample injection volume was 20 mm^3, and the absorbance was monitored at 292 nm.

Chlorophyll content was determined according to Chory *et al.* (1994), ascorbic acid content was quantified as described by Klein and Perry (1982), and total phenols were extracted and measured according to Spanos and Wrolstad (1990). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity in the tobacco leaf was determined spectrophotometrically as described by Gyamfi *et al.* (1999). Similarly, ferric reducing antioxidant power (FRAP) assay was performed to measure the antioxidants or reductants according to Benzie and Strain (1996) and George *et al.* (2004).

Approximately 85 % of the tobacco calli produced leafy structures or a large number of shoot buds after a period of 2 weeks in selection medium. The results presented here are the average of best 10 HPT and TC independent lines and best 5 *HPT:TC* independent lines. The presence of transgenes in T_0 plants was examined by PCR amplification (data not shown). Out of 10 HPT screened, 6 lines revealed the presence of *HPT* gene and out of 10 TC lines, 8 were positive for *TC* and only 3 lines showed the presence of both *HPT* and *TC* genes out of 5 screened (Fig. 1).

The mRNA expression of *HPT* and *TC* genes in transgenic tobacco plants were investigated by RT-PCR analysis using total RNA. The appearance of expected size bands of *HPT* (1.2 kb), *TC* (1.4 kb), and *HPT*:*TC* confirmed the expression of respective genes in the transgenic plants (Fig. 1). These genes did not have the same expression of respective mRNA transcripts. Therefore, the 26S rRNA (500 bp) was used as an internal standard which was equally amplified from every cDNA sample suggesting that cDNA synthesis was done equally from each mRNA sample.

Methanolic extracts of tobacco leaves were subjected to reverse-phase HPLC to measure the tocopherol content. The α-tocopherol content increased 5.4-fold in tobacco leaves of *HPT* transgenic lines in comparison

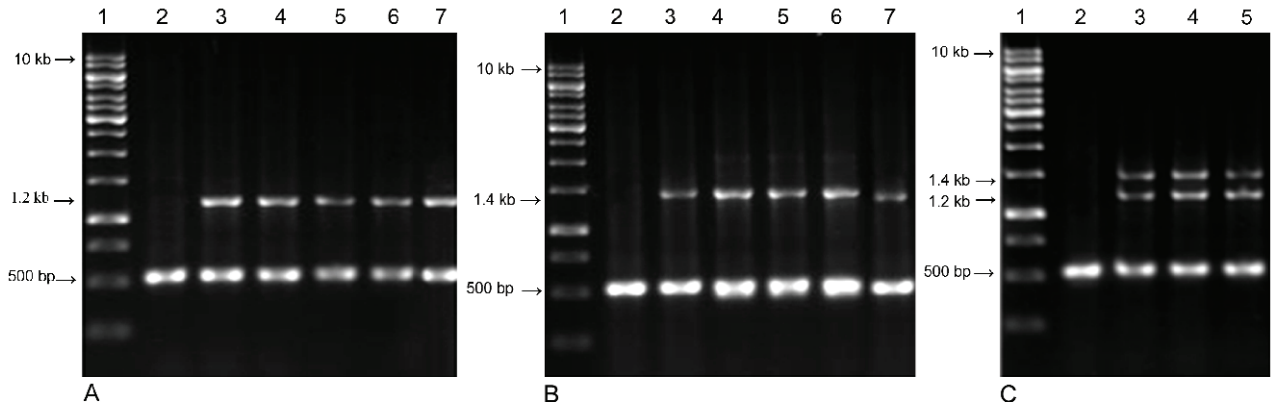

Fig. 1. RT PCR analysis showing mRNA expression pattern of HPT and TC transgenic lines: *A* - *lane* 1 is a 10 kb DNA ladder, *lane* 2 is the wild type expressing only 26SrRNA as an internal reference standard (positive control), *lanes* 3 to 7 correspond to HPT transgenic lines 1, 2, 5, 6, and 9; *B* - *lanes* 3 to 7 correspond to TC transgenic lines 2, 3, 6, 7, and 8; *C* - *lanes* 3 to 5 correspond to double transgenic HPT:TC transgenic lines 1, 3, and 5.

Table 1. Content of α-tocopherol in transgenic tobacco lines overexpressing *HPT, TC*, and *HPT:TC* genes and increase over the control.

Gene	Line	Content [μg g^{-1}(f.m.)]	Increase [fold]
Control		2.24 ± 0.06	-
HPT	1	6.94 ± 0.22	3.1
	2	9.85 ± 0.24	4.4
	4	11.20 ± 0.35	5.0
	5	10.70 ± 0.17	4.8
	6	10.50 ± 0.20	4.7
	9	12.09 ± 0.51	5.4
TC	2	6.04 ± 0.11	2.7
	3	6.04 ± 0.11	2.7
	6	6.72 ± 0.17	3.0
	7	8.96 ± 0.10	4.0
	8	8.28 ± 0.21	3.7
HPT:TC	1	6.04 ± 0.11	2.7
	3	7.16 ± 0.09	3.2
	5	15.90 ± 0.36	7.1

with WT control. Similarly, the *TC* transgenic tobacco lines showed 4-fold increase in α-tocopherol content in the leaves. The α-tocopherol content in double transgenic *HPT*:*TC* lines increased by 7.1-fold (Table 1).

The T_0 lines showing the higher α-tocopherol content were selected for Southern blot analysis to confirm the integration of *HPT* and *TC* genes in the genome, respectively. The expected hybridization signal corresponding to the full-length *HPT* (1.2 kb) and *TC* (2 kb) transgenes were observed as expected. This provided the evidence of stable integration of complete *HPT*, *TC*, and *HPT*:*TC* transgenes in the tobacco plant genome (Fig. 2).

The total chlorophyll content was measured in the 10-week-old tobacco plants (wild and transgenic) using fully expanded leaves. Chlorophyll content in the transgenic tobacco plants were 0.84 μg g^{-1}(f.m.) in *HPT* lines, 0.86 μg g^{-1} in *TC* lines, 0.90 μg g^{-1}(f.m.) in *HPT*:*TC* double transgenic lines, and 0.84 μg g^{-1}(f.m.) in wild type (Table 2). The transgenic tobacco plants showed significantly ($P < 0.05$) higher ascorbic acid content (1.11-fold in *HPT* line, 1.16-fold in *TC* line, and 1.2-fold in *HPT*: *TC* double transgenic line) when compared to the WT (Table 2). The total phenolic content in the transgenic and wild lines are given in the Table 2.

The transgenic tobacco leaves indicated better DPPH radical scavenging activity when compared to the WT (Table 2). The total antioxidant activity (FRAP) was in the order : *HPT*:*TC* > *HPT* > *TC* lines > WT (Table 2).

The present study is an attempt to enhance α-tocopherol in tobacco plants by overexpressing *Arabidopsis HPT* and *TC* genes *via Agrobacterium* mediated genetic transformation. The reason for variation in α-tocopherol content in tobacco lines may be due to difference in the expression of the transgenes, due to the position effect, or due to the availability of substrates. There are two potentially limiting substrates, homogentisic acid (HGA) derived from prephenate of shikimate pathway and phytyl-diphosphate (PDP) derived from phytol. Generally, the tocopherol synthesis is proportional to chlorophyll content, and under stress, chlorophyll degradation correlated with decline in tocopherol content, but still there is a lack of direct evidence for the incorporation of chlorophyll-derived phytol into tocopherols (Rise *et al.* 1989, Collakova and DellaPenna 2003b). It was proved in the feeding experiments in soybean and sunflower suspension cultures that tocopherol biosynthesis was limited by the availability of HGA, phytol, and presumably PDP (Caretto *et al.* 2004, Karunanandaa *et al.* 2005).

The activity of TC depends on the immediate products of HGA and PDP condensation reaction catalyzed by HPT. These products, 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) and 2,3-dimethyl-5-phytyl benzoquinone (DMPBQ), act as the substrates for TC. Thus, the co-expression of these genes in plants should lead to increasing the total pathway flux and so

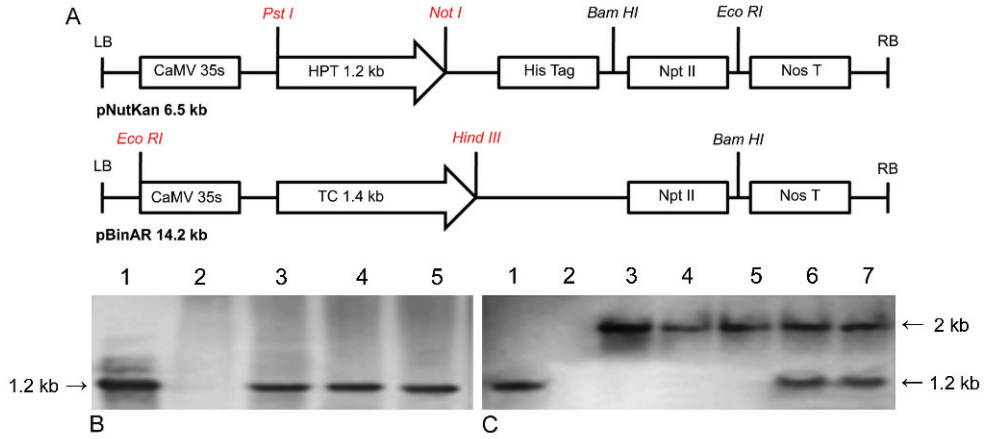

Fig. 2. *A* - Schematic representation of the pNutKan and pBinAR binary vector backbone showing *HPT* and *TC* expression cassette with restriction sites. *B*, *C* - Southern blot analysis of transgenic tobacco HPT, TC, and double transgenic HPT:TC tobacco lines: *B* - *lane* 1 is the positive control: PCR product of *HPT* (1.2 kb) gene, *lane* 2 is wild tobacco DNA not showing any band, *lanes* 3, 4, and 5 show the presence of *HPT* integration in transgenic tobacco lines 2, 4, and 9. *C* - *lane* 1 is the positive control PCR product of *HPT*, *lane* 2 is wild tobacco DNA (negative control), *lanes* 3, 4, and 5 show the presence of *TC* integration in lines 6, 7, and 8, and the *lanes* 6 and 7 show the integration of *HPT* (1.2 kb) and *TC* (2 kb) in double transgenic *HPT*:*TC* lines 1 and 5.

Table 2. Content of chlorophyll, ascorbic acid, total phenolics, DPPH, and FRAP in leaves of wild type and transgenic tobacco plants.

	Chlorophyll [μg g^{-1}(f.m.)]	Ascorbic acid [μg g^{-1}(f.m.)]	Total phenolics [μg g^{-1}(f.m.)]	DPPH [%]	FRAP [mmol g^{-1}(d.m.)]
Wild	0.838 ± 0.019 ^{b}	0.648 ± 0.010 ^{d}	27.34 ± 0.15 ^{a}	26.24 ± 0.621 ^{c}	2.15 ± 0.19 ^{b}
HPT	0.841 ± 0.021 ^{b}	0.721 ± 0.013 ^{c}	27.70 ± 0.30 ^{a}	30.89 ± 0.487 ^{b}	2.61 ± 0.30 ^{a}
TC	0.856 ± 0.008 ^{b}	0.756 ± 0.001 ^{b}	27.07 ± 0.24 ^{a}	30.52 ± 0.339 ^{b}	2.43 ± 0.18 ^{b}
HPT:TC	0.904 ± 0.008 ^{a}	0.784 ± 0.002 ^{a}	28.02 ± 0.10 ^{a}	37.56 ± 0.404 ^{a}	2.99 ± 0.19 ^{a}

increasing the amount of end products.

Our results are in accordance with the earlier reports that the transgenic expression of *Arabidopsis thaliana HPT* (*AtHPT*) in *Arabidopsis* and other host plants resulted in increased tocopherol content. For tocopherol biosyn-thesis, this reaction is catalyzed by HPT. Indeed, transgenic expression of *Arabidopsis thaliana HPT* (*AtHPT*) in *Arabidopsis* and other host plants resulted in increased tocopherol content. In *Arabidopsis*, the total leaf tocopherol increased up to 4.4-fold (Collakova and DellaPenna 2003a). Interestingly, the *AtHPT* expression in *Arabidopsis* was surpassed by the environmental factors, such as high irradiance and nutrient deficiency which resulted in 18-fold increased leaf tocopherol content (Collakova and DellaPenna 2003b). This observation either might indicate that the potential of *AtHPT* overexpression was not utilized to its full extent under non-stressed conditions, or may be due to the heterologous expression of the genes in tobacco, or due to factors contributing to tocochromanol flux regulation. Recently, Chen *et al*. (2012) has found that overexpressed γ-tocopherol methyltransferase from *Brassica napus* (*BnTMT*) in soybean under the control of a seed specific promoter from *Arabidopsis* fatty acid elongase I (*FAE I*) resulted in 11.1- and 18.9-fold increase in α- and β-tocopherols.

Overexpression of 4-hydroxyphenylpyruvate dioxy-genase (*HPPD*) gene targeted to tobacco cytosol or plastids shows a modest increase of α-tocopherol in seeds (Falk *et al.* 2003, 2005). Interestingly, Karunanandaa *et al.* (2005) and Rippert *et al.* (2004) found that combined expression of HPPD and a prephenate dehydro-genase or a bifunctional prephenate dehydrogenase led to the accumulation of substantial tocotrienol suggesting that PDP was limiting for tocopherol biosynthesis in plants with high HGA content.

Li *et al.* (2011) has reported the strategy to improve tocopherol content by overexpressing the multiple genes such as *HPT* and γ-tocopherol methyl transferase (γ-*TMT*) in lettuce (dual transgenic) with 9-fold increase in tocopherol which is very similar to our results. Lee *et al.* (2007) reported the 2.7- and 2.0-fold increase in α-tocopherol content in lettuce leaves by overexpressing *Arabidopsis HPT* and *TC*. Similarly, Seo *et al.* (2011) expressed a *HPT* homologue (*MdHPT1*) from apple fruits in tomato and α-tocopherol content in leaves and fruits increased up to 3.6- and 1.7-fold, respectively. In the case of co-expression of two genes (*HPT*:*TMT*), Collakova and DellaPenna (2003a,b) reported nearly 3.2- and 12-fold increase in α-tocopherol content in *Arabidopsis* leaves and seeds, respectively. Similarly, the expression

of *Arabidopsis TyrA* (coding tyrosine amino transaminase) combined with the overexpression of *HPPD* resulted in 8-fold increase in α-tocopherol content (Rippert *et al.* 2004). Karunanandaa *et al.* (2005) have reported 10.4 to 11.5-fold increases in the tocopherol content in the soybean seeds when co-expressed *Arabidopsis HPPD, HPT*, and geranylgeranyldiphosphate hydratase (*GGH*) in *Erwinia herbicola* seeds. In plants, tocopherol composition varies between the different species and tissues within the species (Grusak and DellaPenna 1999). Usually, leaves accumulate more α-tocopherol, whereas seeds are rich in γ-, β-, and δ-tocopherols.

The overexpression of *Arabidopsis HPT* and *TC* genes significantly altered the content of chlorophylls and antioxidants, and activities of antioxidative enzymes in transgenic tobacco (Marcel *et al.* (2001). UV and oxidative stress induced accumulation of the phenolic compounds and flavonoids resulting in increased DPPH and FRAP which have strong correlation with the production of ROS scavenging radicals during stress conditions (Majer *et al.* 2010).

This study highlights the importance of enhancing vitamin E content in crop plants in order to meet the future nutritional challenges.

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