Transgenic Res
DOI 10.1007/s11248-007-9091-1

ORIGINAL PAPER

Accumulation of vitamin E in potato (*Solanum tuberosum*) tubers

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Received: 28 January 2007 / Accepted: 3 March 2007

Abstract Vitamin E (tocopherol) is a powerful antioxidant essential for human health and synthesized only by photosynthetic organisms. The effects of over-expression of tocopherol biosynthetic enzymes have been studied in leaves and seeds, but not in a non-photosynthetic, below-ground plant organ. Genetic and molecular approaches were used to determine if increased levels of tocopherols can be accumulated in potato (*Solanum tuberosum* L.) tubers through metabolic engineering. Two transgenes were constitutively over-expressed in potato: *Arabidopsis thaliana* p-hydroxyphenylpyruvate dioxygenase (At-HPPD) and *A. thaliana* homogentisate phytyltransferase (At-HPT). α-Tocopherol levels in the transgenic plants were determined by high-performance liquid chromatography. In potato tubers, over-expression of At-HPPD resulted in a maximum 266% increase in α-tocopherol, and over-expression of At-HPT yielded a 106% increase. However, tubers from transgenic plants still accumulated approximately 10- and 100-fold less α-tocopherol than leaves or seeds, respectively. The results indicate that physiological and regulatory constraints may be the most limiting factors for tocopherol accumulation in potato tubers. Studying regulation and induction of tocopherol biosynthesis should reveal approaches to more effectively engineer crops with enhanced tocopherol content.

Keywords *p*-Hydroxyphenylpyruvate dioxygenase · Homogentisate phytyltransferase · α-Tocopherol · Metabolic engineering

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Introduction

Vitamin E is an essential nutrient for human health, but is consumed at suboptimal levels. The importance of vitamin E for reproductive health was recognized as early as 1922 (Brigelius-Flohé and Traber, 1999). Vitamin E is the collective term for eight structurally related tocochromanol compounds, four tocopherols (α, γ, β, and δ) and four tocotrienols (α, γ, β, and δ), which differ from one another based on the number and position of methyl groups on the chromanol ring. α-Tocopherol appears to be the most biologically active in

humans, and is the most powerful lipid-soluble antioxidant known (Schneider, 2005). Evidence suggesting important roles for the other isomers, notably tocotrienols and γ-tocopherol, has recently emerged (Jiang et al., 2004; Kamal-Eldin and Appelqvist, 1996). In addition to serving as a potent antioxidant, α-tocopherol has also been shown to act as a signaling molecule in muscle tissue (Ricciarelli et al., 1998). Humans and other animals are not capable of synthesizing tocopherols or tocotrienols autonomously and must obtain them from their diet. Approximately 90% of children and adults in the United States do not consume the recommended amount of vitamin E (Drewel et al., 2006; Ahuja et al., 2004).

The first committed step of tocopherol biosynthesis is the condensation of the aromatic head group of homogentisic acid with isoprenoid-derived phytyldiphosphate, catalyzed by homogentisate phytyltransferase (HPT) (Cheng et al., 2003) (Fig. 1). Tocotrienols are synthesized by

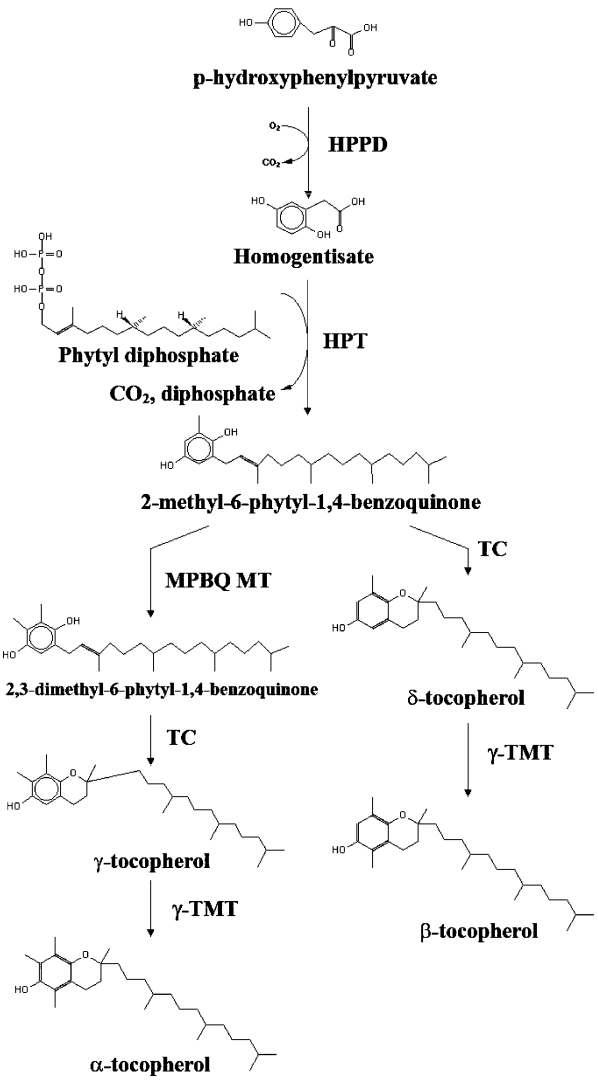

Fig. 1 Tocopherol biosynthesis in cyanobacteria and higher plants. Homogentisate is derived from the shikimate pathway, either via prephenate or through transamination of tyrosine (Garcia et al., 1999). Phytyl diphosphate originates from the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway through reduction of geranylgeranyl diphosphate (Eisenreich et al., 1998; Lichtenthaler, 1998). Figure is derived from AraCyc (Mueller et al., 2003)

the condensation of homogentisic acid with a different prenyl-diphosphate, geranylgeranyldiphosphate. The enzymes involved in tocochromanol synthesis are all membrane-bound and localized to the chloroplast inner membrane, with the exception of p-hydroxyphenylpyruvate dioxygenase (HPPD), which has been shown to be cytosolic (Garcia et al., 1999). HPPD, encoded by the *Arabidopsis* gene *PDS1*, catalyzes the conversion of p-hydroxyphenylpyruvate to homogentisic acid.

In plants, the function of tocopherols remains unclear. The accumulation of tocopherols in leaf plastid membranes suggests a role in scavenging free radicals generated from photo-oxidation (Havaux et al., 2005). In cyanobacteria, tocopherols protect membranes from peroxidation (Maeda et al., 2005), a function that has been shown to be conserved for germinating seeds (Sattler et al., 2004). A perplexing role in sucrose transport is also apparent, since tocopherol-deficient plants fail to export photoassimilate from leaves (Provencher et al., 2001; Hofius et al., 2004). The different isomers of tocopherols and tocotrienols may also have unique functions in plants, as suggested from their distribution in different tissues. In general, seeds accumulate high levels of γ-tocopherol and leaves accumulate a higher percentage of α-tocopherol, while the remaining isomers are not naturally abundant. Tocotrienols are abundant in the seeds of most monocots, and rarely found in dicots (Cahoon et al., 2003).

In recent years, all of the genes in the vitamin E pathway have been cloned, many of which were identified using a genomics approach based on sequence homology between the model plant *Arabidopsis thaliana* and the cyanobacterium *Synechocystis* (Shintani and DellaPenna, 1998). These discoveries precipitated a large number of genetic studies that have added to our understanding of the regulation of the pathway, and demonstrated the potential to increase accumulation and improve the composition of vitamin E in crops by metabolic engineering (see DellaPenna, 2005 for a recent review). Because of its apparent higher activity in mammalian cells, α-tocopherol has been the major target for enhancement in crops. While over-expression of γ-tocopherol methyltransferase and/or 2-methyl-6-phytylbenzoquinol methyltransferase has proven sufficient to successfully modify composition of tocochromanols in crop plants (Shintani and DellaPenna, 1998; Van Eenennaam et al., 2003), increasing levels of tocopherols have been shown to be far more challenging (DellaPenna, 2005). Efforts to enhance total tocopherol levels have met with limited success, but have raised important questions regarding the regulation of the tocopherol biosynthetic pathway. Studying the development- and tissue-dependent regulation of vitamin E synthesis may suggest more effective approaches for engineering enhanced vitamin E content in crop plants. Since vitamin E synthesis has only been studied in leaves and seeds thus far, potato tuber was used as a model for studying the accumulation of vitamin E and the regulation of its synthesis in a below-ground, non-photosynthetic plant organ.

The potato (*Solanum tuberosum* L.) is an important vegetable and staple food, with world production averaging over 311 million metric tons in 2003 (National Potato Council, 2006). Highly nutritious, potato is an excellent target for nutritional improvement. Because of the high yield and low cost of cultivation, potatoes are an important part of the diet in many cultures and nutritional improvement of potato would have a significant impact on vitamin and nutrient intake for these populations. Thus far, research to metabolically engineer potato for enhanced nutritional value has been confined to the production of specific carotenoids. For example, over-expression of phytoene synthase was shown to result in 19-fold higher levels of lutein, as well as higher β-carotene levels (Ducreux et al., 2005), zeaxanthin contents were increased up to 130-fold through disruption of zeaxanthin catabolism (Römer et al., 2002), and accumulation of astaxanthin was achieved through expression of a microbial β-carotene ketolase gene (Morris et al., 2006).

In this study, HPPD and HPT were over-expressed to assess their impact on vitamin E accumulation in potato tubers. To our knowledge, this is the first investigation of vitamin E biosynthesis in an underground, non-photosynthetic tissue and the first attempt to elevate vitamin E levels in potato leaves and tubers.

Materials and methods

Plant material and growing conditions

Potato genotypes 'Spunta' and MSE149-5Y were propagated in 25 × 150 mm culture tubes in General Propagation Medium (Yadav and Sticklen, 1995). Tissue culture-propagated plants were planted in the greenhouse in 10 cm pots and subsequently transferred to 19 L pots after one month. Selected lines for characterization were grown together in six replicates from February to May with untransformed control plants. Location of the plants in the greenhouse was randomized for all experiments. Tubers were protected from light and stored at room temperature for two days before storage at 4°C.

Plasmid engineering and plant transformation

cDNAs of genes encoding *A. thaliana* HPPD (*PDS1*, referred to as At-HPPD) and HPT (*HPT1*, referred to as At-HPT) were kindly provided by Dr Dean DellaPenna (Department of Biochemistry, Michigan State University) (Savidge et al., 2002; Norris et al., 1998). Both genes were sub-cloned downstream of double 35S CaMV promoter and upstream of nopaline synthase terminator in the plant transformation vector pSPUD4, a derivative of pBI121 (Clontech, Palo Alto, CA, USA). A 1,737 bp *Bam*HI-*Xba*I fragment containing the HPPD open-reading frame was sub-cloned into pSPUD4 to generate pHPPD. The HPT coding sequence was PCR-amplified and cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA); subsequently a 1,577 bp *Bam*HI fragment was sub-cloned into pSPUD4 to construct pHPT. The sequenced plant transformation vectors were introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation, using a Bio-Rad (Hercules, CA, USA) electroporator, and cuvettes according to the manufacturer's instructions. Primers for cloning, PCR, and RT-PCR are listed in Table 1.

Published protocols for potato transformation were used (Douches et al., 1998). Transgenic shoots were selected on General Propagation Medium containing 50 mg/L kanamycin.

High performance liquid chromatography (HPLC)

Tuber tocopherol levels were first screened using two plant replicates and two to three pooled tubers. The lines with the highest levels were further characterized using five plant replicates and three to four tubers per replication. Measurements on control plants were replicated five times in all experiments. Method for extraction of total lipids from potato tuber was developed

Table 1 Primer sequences for PCR

Purpose		Sequence (5′ to 3′)
Amplify HPT1 from plasmid DNA	forward	CACACCGAACCTTAAGATTTTGC
	reverse	GCTCTAGAACTAGTGGATCC
Confirm presence of transgenes		
A.t. HPPD (PDS1)	forward	GGACCCCCACCCACGAGGAGCAT
	reverse	AATTTAGCAGCAGCGGTTTCTTTCATA
A.t. HPT1	forward	GGACCCCCACCCACGAGGAGCATC
	reverse	CCCACCAGCAGCGGAAACAAGAGAAGAAC
nptII	forward	AGCAGCCAGTCCCTTCCCGCTTCA
	reverse	CCACCCAAGCGGCCGGAGAACCTGCG
Real-time RT–PCR		
A.t. HPT1	forward	GAGTTCTGCGTTGTGATTCGAGTAAAGTT
	reverse	ACAGCCTCCAAGATGCCAGTGAAAAGTAA
S.t. HPT	forward	TCTCGCGGCCCCATACCATAATAGG
	reverse	ACGATCCATCCAAGCCAAAAACTCAAAAT
A.t. HPPD (PDS1)	forward	GGAGAGATTAAACCGACAACCACAG
	reverse	CGACGACGCATCCTCTACACGC
S.t. Beta-tubulin	forward	AGGGACTGGATCTGGCATGGGAACACTAC
	reverse	CAGGGAATCTCAAACAGCAAGTAACAC

based on published protocols (Römer et al., 2002; Sattler et al., 2003). Tubers showing traces of green were excluded. Total lipids were extracted from approximately 0.5 g of fresh tuber homogenized in a commercial blender, exact tissue mass was recorded, and the sample was immediately suspended in 2.5 ml extraction buffer (methanol:chloroform 2:1, BHT 1 mg/ml, and tocol 1.25 μg/ml). Two milliliters of Tris-HCl (50 mM, pH 7.5) and 500 μl of chloroform were added. After vortexing for 2 min and centrifugation at 3,000 × *g* for 10 min, the dried residue from the organic layer was resuspended in 100 μl acetonitrile. Fifty microliters were injected for HPLC analysis on a Spherisorb ODS-2 5 μm, 250 × 4.6 mm reverse-phase column. Tocopherols were detected by fluorescence using 290 nm excitation and 325 nm emission, identified by retention times, and quantified by integrating peak area relative to nanograms of tocopherol in standard curve samples. Tocopherol standards and tocol internal standard were obtained from Matreya (Pleasant Gap, PA, USA).

Leaf tocopherol levels were determined using five plant replicates and pooled leaves. Leaf tocopherol content was determined by extracting total lipids from 30 mg of pooled leaf tissue (modified from Sattler et al., 2003). The tissue was ground in liquid nitrogen, and extracted as above with proportionately adjusted volumes of buffer and solvent. The resuspended residue was injected as for tuber samples. Only newly expanded, healthy, unshaded leaves were used for analysis.

Polymerase chain reaction (PCR) and DNA isolation

Primers were designed using DNA Star (DNA Star Inc., Madison, WI, USA), or by visual examination of sequences and ordered from IDTDNA (Coralville, IA, USA) (Table 1). Primers for PCR were used at a final concentration of 25 pmol/μl (Invitrogen, (Carlsbad, CA, USA) *Taq* polymerase, buffer, and $MgCl_2$ were used according to the manufacturer's instructions.

Plant genomic DNA was isolated using Qiagen (Valencia, CA, USA) DNA Easy kit according to manufacturer's instructions, or modified CTAB extraction method (Monsanto Biotechnology Regulatory Services, 2004). For CTAB extraction, 2 g of leaf tissue was ground in 7 ml of extraction buffer (0.1 M Tris-HCl pH 8, 1.4 M NaCl, 0.02 M EDTA pH 8, 2% hexadecyltrimethylammonium bromide (w/v) proteinase K (1 mg/100 ml)), and incubated at 55°C for 1 h. Seven milliliters phenol:chloroform:isoamyl alcohol (25:24:1 v/v) were added and mixed by inversion, samples were centrifuged at 3,000 × *g* for 10 min, 6 ml of supernatant were transferred to a new tube, and the phenol:chloroform extraction was repeated three times. Supernatant was incubated for 30 min at room temperature in 50 μl RNaseA (10 mg/ml) before the final extraction. DNA was precipitated using isopropanol, centrifuged, and the pellet washed in 2 ml of 70% ethanol before resuspension in 1 × Tris-EDTA.

Real-time RT-PCR

Primers to amplify *S. tuberosum* HPT (St-HPT) were designed based on consensus cDNA sequences obtained from The Institute for Genomic Research Potato Gene Index (TIGR Gene Index Databases, 2004) (Table 1). Primers used to compare transcript levels of transgenes were designed to anneal to transgene sequences dissimilar from endogenous homologues by examining aligned sequences using BLAST (Altschul et al., 1990).

RNA from 30 mg of leaf tissue was extracted using the Machery-Nagel plant RNA kit according to the manufacturer's instructions. Following spectrophotometric quantification, first-strand cDNA synthesis was performed with M-MLV RT (Invitrogen, Carlsbad, CA, USA) and gene-specific primers (Table 1). Manufacturer's protocol was modified as follows: following denaturation at 65°C, the reaction was incubated at the average optimal annealing temperature for the primers, then chilled on ice before addition of M-MLV RT.

Real-time measurements were made using a MX4000 thermal cycler (Stratagene, La Jolla, CA, USA) and SYBR green with ROX reference dye. Comparative threshold (C_t) values were normalized to *β*-tubulin internal control and compared to obtain relative expression levels. In reactions to

which no reverse transcriptase had been added, fluorescence was below background levels (no C_t). All experiments were conducted with two to three plant replicates, from which RNA was extracted and cDNA synthesized independently.

Southern analysis

Southern analysis was performed according to the method of McGrath et al. (1993). Total plant genomic DNA extracted using CTAB was digested overnight using *Eco*RI restriction enzyme (New England Biolabs, Beverly, MA, USA). Approximately 10 μg of the restricted fragments were separated on a 0.8% agarose gel at 3 V/cm and using TAE running buffer (40 mM Tris-acetate, 1 mM EDTA). A 366 bp DNA fragment of the *npt*II gene was amplified from pHPT plasmid DNA (P1: ACCTTGCTCCTGCCGAGAAAGTAT; P2: TATCACGGGTAGCCAACGCTATGT). Approximately 50 μg of probe DNA were labeled overnight using a Random Primers DNA Labeling kit. Residual unincorporated nucleotides were removed by purification through a Sephadex column. A final low stringency wash was performed using 2 × SSC, 0.1% SDS. The blot was exposed to Hyperfilm MP film (Amersham Biosciences UK Limited, Buckinghamshire, UK) for 33 h at −80°C and developed.

Statistical analysis

Results from experiments were analyzed using PROC GLM program of SAS (SAS Institute, 2005). Statistically significant differences were determined using a one-way ANOVA and by making pair-wise comparisons between least square means. Means were considered significantly different at α level of 0.05.

Results

Transformation of potato

To test the individual effects of HPPD and HPT levels on tocopherol accumulation in potato tubers, the *Arabidopsis* genes At-HPPD and At-HPT were over-expressed in potato using the constitutive CaMV 35S promoter. *Agrobacterium*-mediated transformation resulted in the production of shoots from cv. Spunta and MSE149-5Y. Selection for kanamycin resistance resulted in the identification of 38 Spunta pHPPD-transformed (SpHPPD) lines, seven Spunta pHPT-transformed (SpHPT) lines, 30 MSE149-5Y pHPPD-transformed (EHPPD) lines, and three MSE149-5Y pHPT-transformed (EHPT) lines. From among these transformants, 10 SpHPPD, 11 EHPPD, 7 SpHPT, and 3 EHPT lines were used for further study. PCR, using transgene-specific primers, verified the presence of At-HPPD or At-HPT in all transgenic lines studied (data not shown). Primers for amplification of transgenes did not produce amplification products from genomic DNA of untransformed plants.

Tocopherol content in SpHPPD transgenic potato leaves

Leaf α-tocopherol levels were shown to be significantly higher than levels in cv. Spunta in four transgenic SpHPPD lines tested ($P < 0.01$) (Fig. 2).Untransformed plants of cv. Spunta and MSE149-5Y were not significantly different from one another. Percent increase in α-tocopherol ranged from 41 to 112%, with cv. Spunta accumulating 7.4 ± 0.4 μg of α-tocopherol per gram fresh weight (gfw) and transgenic plants accumulating 11.9 ± 1.8 μg/gfw on average.

Molecular characterization of HPPD and HPT transgenic lines

Southern analysis was conducted on eight high tocopherol lines, as well as four low/wild-type tocopherol lines. Integration of the At-HPPD or At-HPT transgene was inferred by Southern analysis of genomic DNA using a probe against the *npt*II selectable marker gene. The *npt*II probe did not hybridize to genomic DNA from cv. Spunta or MSE149-5Y, but produced a detectable signal in lanes containing pHPPD or pHPT plasmid DNA affixed to the membrane. Ten of the lines tested contained a single copy of the transgene, while SpHPT3 and SpHPT8 had two integrated copies (data not shown).

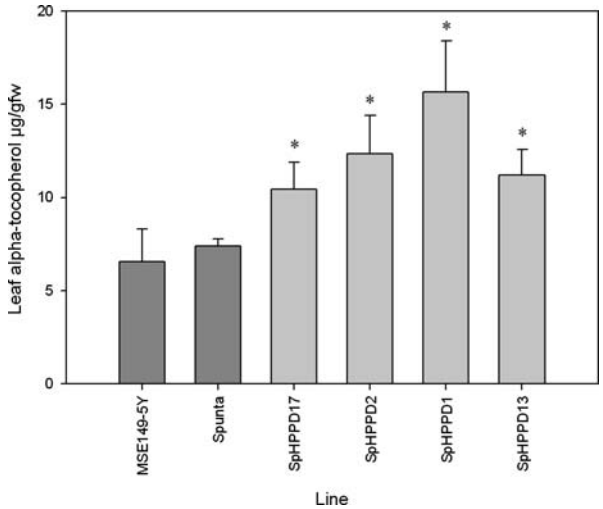

Fig. 2 Comparison of α-tocopherol levels in leaves of four SpHPPD transgenic lines and untransformed controls. Tissue culture-derived plants were grown in the greenhouse for two months prior to quantification of α-tocopherol by HPLC. Each line is represented as an average±SD of measurements of five plants using pooled leaves. Lines significantly different from untransformed cv. Spunta are marked (*)

Real-time RT-PCR was used to compare the expression level of At-HPPD in the four selected transgenic lines, SpHPPD13, SpHPPD15, EHPPD6, and EHPPD12. Products of RT-PCR were verified by sequencing, by examining dissociation curves, and by electrophoresis of the product on 0.8% agarose gel. The primer sets used did not amplify genomic DNA from untransformed plants. The RNA preparations were shown to be free of contaminating genomic DNA since reactions containing no reverse transcriptase produced no amplification, and the dissociation curves generated from amplification of the cDNA showed a single, distinct product. High levels of the At-HPPD transcript were detected in all four transgenic lines, with low variability between lines (Fig. 3a).

Some At-HPT transformants appeared to have lower levels of tuber α-tocopherol than control plants, although these decreases in α-tocopherol were not statistically significant (data not shown). The possibility of co-suppression was tested by determining transcript levels of At-HPT and the potato homologue St-HPT by real-time RT-PCR in seven transgenic lines. High levels of At-HPT transcript were detected in all seven lines, including those that had lower mean α-tocopherol levels than controls (Fig. 3b). The endogenous potato St-HPT had lower steady-state RNA levels than the transgenic At-HPT. Variation in levels of St-HPT expression between lines was low, but Spunta transformants appeared to express the endogenous HPT at slightly higher levels than MSE149-5Y transformants.

Tocopherol content in transgenic potato tubers

Tubers from transgenic plants over-expressing At-HPPD or At-HPT did not exhibit changes in tocopherol composition compared with untransformed controls (data not shown). The majority of tocopherols were accumulated as α-tocopherol, γ-tocopherol was barely detectable, and β- and δ-tocopherol were not detected. For all subsequent experiments, only α-tocopherol levels were quantified. Two lines of each genotype/vector combination that exhibited the highest α-tocopherol levels were selected for full characterization.

Tubers harvested from four selected individual Spunta transformants did not significantly differ in α-tocopherol levels compared with tubers from untransformed control plants (Table 2), despite high transgene expression levels in these lines. The two characterized SpHPT lines accumulated

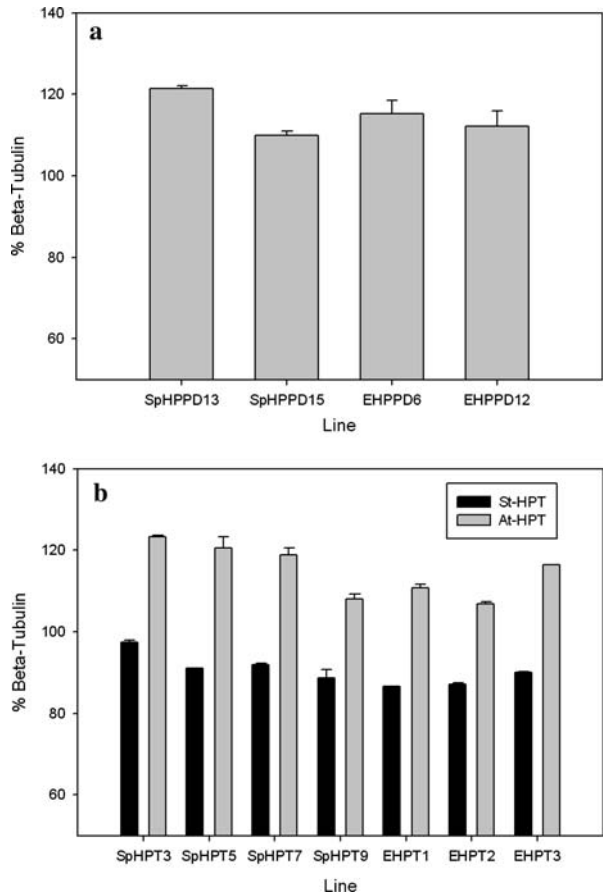

Fig. 3 Comparison of relative transcript levels of At-HPPD (a) and At-HPT and St-HPT (b) among characterized transgenic lines. Real-time RT-PCR comparative threshold values were normalized to β-tubulin internal control and plotted. Each line represents the average±SD of two measurements. Products of real-time RT-PCR were electrophoresed on 0.8% agarose gel to verify amplification specificity

Table 2 Comparison of α-tocopherol content in tubers of HPPD and HPT transformants and untransformed controls

	Mean α-tocopherol			
	(ng/gfw)	SD	*P*-value	% change
cv. Spunta	432.9	165.8		
SpHPT3	458.6	53.4	0.7750	6
SpHPT7	506.7	99.4	0.4131	17
SpHPPD13	338.5	145.0	0.2969	–22
SpHPPD15	367.4	147.0	0.4674	–15
MSE149-5Y	281.5	114.4		
EHPT1	413.1	64.1	0.1483	47
EHPT2	578.6	109.9	0.0019	106
EHPPD6	502.1	136.4	0.0178	78
EHPPD12	1030.7	261.8	<0.0001	266

Values represent the mean of five measurements of pooled tubers. All *P*-values and percent change represent comparisons to the respective untransformed genotype

459 and 507 ng/gfw of α-tocopherol in their tubers, values that were slightly higher than cv. Spunta mean (433 ng/gfw). Mean tocopherol levels of the two characterized SpHPPD transformants were 338 and 367 ng/gfw of α-tocopherol, slightly lower than the control mean (433 ng/gfw).

In contrast to results in the Spunta background, three of the four transformants of MSE149-5Y had significantly higher levels of tuber α-tocopherol than the control (Table 2). In MSE149-5Y transgenic plants over-expressing At-HPT, α-tocopherol levels increased from 208 to 579 ng/gfw. In plants over-expressing At-HPPD, α-tocopherol levels increased from 208 to 1030 ng/gfw. Compared with MSE149-5Y,

EHPT2 accumulated significantly higher levels of α-tocopherol, representing an increase of 106% with a mean of 579 ng/gfw ($P = 0.0019$). EHPT1 exhibited higher levels of α-tocopherol at 413 ng/gfw, but was not significantly different from the control ($P = 0.1483$). EHPPD6 had a mean of 502 ng/gfw, representing a significant 78% increase over the control mean ($P = 0.0178$). The best performing line was EHPPD12, which showed a 266% increase over control values, and accumulated 1030 ng of α-tocopherol per gram of fresh weight tuber tissue ($P < 0.0001$).

Discussion

HPPD catalyzes conversion of p-hydroxyphenylpyruvate to homogentisic acid. HPPD has been predicted to be a regulatory enzyme of tocopherol biosynthesis for numerous reasons: the enzyme catalyzes one of the first committed steps in tocopherol biosynthesis; increased At-HPPD expression in senescing leaves is associated with tocopherol accumulation; and feeding safflower cell cultures with exogenous HGA leads to increased tocopherol accumulation (Tsegaye et al., 2002). HPT catalyzes condensation of homogentisic acid with isoprenoid-derived phytyldiphosphate in the first committed reaction step of tocopherol biosynthesis, and thus may also represent an important regulatory step (Savidge et al., 2002).

Three lines of evidence indicate that over-expression of At-HPPD and At-HPT cause the increases in tuber α-tocopherol observed in EHPT2, EHPPD6, and EHPPD12. At-HPPD and At-HPT transgenes are stably integrated into the genomes of these transgenic lines as shown by Southern analysis. At-HPPD and At-HPT are expressed at high levels in EHPT2, EHPPD6, and EHPPD12. Finally, the increases in α-tocopherol in these three transgenic lines are statistically significant.

Because of the heterogeneous nature of potato cultivars, two different genotypes were selected for *Agrobacterium*-mediated transformations with vectors pHPPD and pHPT. Phenotype of cv. Spunta and MSE149-5Y transformants differed greatly, with MSE149-5Y showing greater increase in tocopherol accumulation. Untransformed cv. Spunta and MSE149-5Y have similar endogenous tocopherol levels, but are not related in parentage. The reason for disparity in the effect of over-expression of HPPD or HPT in these two genotypes remains unknown, and may reflect a difference in flexibility of isoprenoid metabolism, abundance of precursors, or other factors.

Over-expression of 35S::At-HPT results in increases in total tocopherols of three-fold to 4.4-fold in *Arabidopsis* leaves and 40% in *Arabidopsis* seeds (Collakova and DellaPenna, 2003). A maximum of two-fold increase in homozygous seed was obtained when At-HPT was expressed with a seed-specific promoter (Savidge et al., 2002). Potato tubers from HPT transformants accumulated 106% higher levels of α-tocopherol, indicating that over-expression of At-HPT also leads to increased tocopherol synthesis in potato tuber tissue. When expressed under control of the 35S CaMV promoter, At-HPT causes a proportionately greater increase in α-tocopherol in tubers than in seeds, but far smaller than that observed in leaves.

Over-expression of At-HPPD in the cyanobacteria *Synechocystis* leads to a seven-fold increase in tocochromanols (Karunanandaa et al., 2005). However, in transgenic *Arabidopsis* showing 10-fold increases in HPPD activity, leaf tocopherol levels were only 15–37% elevated over wild-type levels (Tsegaye et al., 2002). In this same study, 9- to 17-fold higher HPPD protein levels in the seeds from transgenic plants translated to an unexpectedly small 24–28% increase in tocopherols. Results from quantification of tocopherols in leaves of potato plants over-expressing At-HPPD roughly agreed with these previous reports, showing increases in the range of 41–112%. In tuber tissue, over-expression of At-HPPD seems to have a proportionately greater effect on tocopherol accumulation than in leaves or seeds. Two individual MSE149-5Y transformants over-expressing At-HPPD showed highly significant increases in tuber α-tocopherol of 78% and 266%, respectively.

Increases in α-tocopherol in potato tuber were compared with results in *Arabidopsis* over-expressing At-HPPD and At-HPT under control of the same promoter (Table 3). Nanograms of

Table 3 Comparison of tocopherol content in HPT- and HPPD-over-expressing *A. thaliana* and *S. tuberosum* plants

		pmol/mg Fresh weight		% Increase over wt	Additional pmol/mgfw	% H_2O[a]	pmol/mg Dry weight		Reference
HPT	*A. thaliana*	Wild-type	Transgenic				Wild-type	Transgenic	
	Seed	838.00	1148.00	37	310.00	11.00	941.57	1289.89	Collakova and DellaPenna, 2003
	Leaf	20.00	90.00	440	70.00	92.00	250.00	1125.00	Collakova and DellaPenna, 2003
	S. tuberosum	Wild-type	Transgenic				Wild-type	Transgenic	
	Tuber	0.65	1.34	106	0.69	79.34	3.16	6.50	This report
HPPD	*A. thaliana*	Wild-type	Transgenic				Wild-type	Transgenic	
	Seed	951.91	1225.87	28	273.96	11.00	1069.56	1377.38	Tsegaye et al, 2002
	Leaf	20.00	27.40	37	7.40	92.00	250.00	342.50	Tsegaye et al, 2002
	S. tuberosum	Wild-type	Transgenic				Wild-type	Transgenic	
	Leaf	17.14	25.97	63	8.83	92.00	214.31	324.67	This report
	Tuber	0.65	1.17	78	0.51	79.34	3.16	5.64	This report
	Tuber	0.65	2.39	266	1.74	79.34	3.16	11.58	This report

[a] Data on percentage water of raw potato from USDA (2005)

tocopherol were converted to picomole (pmol) to facilitate comparison with other reports. Leaves of *Arabidopsis* and potato accumulate similar levels of tocopherols (20 pmol/mg of fresh weight (mgfw) and 17 pmol/mgfw, respectively). Over-expression of At-HPPD results in similar increases in tocopherol content in the two species, with accumulation of an additional 7.4 pmol/mgfw in *Arabidopsis* and an additional 8.83 pmol/mgfw in potato. Based on these results, it is probable that the flux coefficient of HPPD and the availability of precursors for tocopherol synthesis in *Arabidopsis* and potato leaves are similar, indicating that there are no significant species-specific differences for tocopherol synthesis in potato and *Arabidopsis* leaves.

To compare tocopherol content of tubers, leaves, and seeds, these tissues were compared on a dry weight basis to adjust for differing water contents (Table 3). Tocopherol contents in leaves and seeds of *Arabidopsis* over-expressing 35S::At-HPPD are 342.50 pmol/mgdw and 1377.38 pmol/mgdw, respectively, whereas tocopherol content in tubers of potato over-expressing At-HPPD is a maximum of 11.58 pmol/mgdw. Tocopherol contents in leaves and seeds of *Arabidopsis* over-expressing 35S::At-HPT are similar values in the range of 1200 pmol/mgdw, but only 6.5 pmol/mgdw in potato tubers. These results suggest the presence of a metabolic block in tuber tissue that may be related to availability of precursors, regulatory constraints, or the physiological capacity of tuber tissue for accumulation of tocopherols.

Tocopherols accumulate in plastid or thylakoid membranes, where they are purported to protect lipids from reactive oxygen species generated from photosynthesis (Peñuelas and Munné-Bosch, 2005). Potato tubers are non-photosynthetic and very low in lipids (Klaus et al., 2004), precluding the physiological requirement for high levels of tocopherols and perhaps limiting their capacity for accumulation. Over-expression of At-HPPD and At-HPT lead to increased tocopherol accumulation in potato tubers, but HPPD and HPT levels are clearly not the only limiting factors.

As the role of tocopherols in plants begins to be elucidated, a complex regulatory network coordinating tocopherol synthesis with

metabolism of chlorophyll and plastoquinone is becoming apparent. The discovery of a phytol kinase that recycles chlorophyll to phytyldiphosphate adds another element to this metabolic network (Valentin et al., 2006; Ischebeck et al., 2006). We observed that mildly green tubers exhibit greater than 10-fold increases in α-tocopherol levels compared to tubers with no apparent photosynthetic activity (data not shown). This observation suggests that actively photosynthesizing chloroplasts may provide sites and/or precursors for tocopherol biosynthesis normally lacking in potato tuber. Alternatively, transcription factors required for chloroplast development such as GOLDEN2-LIKE (Fitter et al., 2002) may influence expression of tocopherol biosynthetic genes. Finally, precursors such as phytyl diphosphate may be limiting in non-photosynthetic tissues. Geranylgeranyl diphosphate reductase (GGDR) is involved in both tocopherol and chlorophyll biosynthesis, and while GGDR is known to be highly expressed in leaves, its expression level in potato tuber is unknown and may be limiting.

While modifying the tocopherol pool composition appears relatively straight-forward (Shintani and DellaPenna, 1998; Van Eenennaam et al., 2003) increasing the total content of tocopherols in plants has proven far more challenging. As in the majority of metabolic networks studied to date, no single enzyme appears to represent a major bottleneck for tocopherol biosynthesis. In the absence of knowledge of pathway regulation or flux, the most fruitful strategy to engineer enhanced vitamin E levels will likely employ tissue-specific over-expression of multiple pathway genes. Our results agree with those of Karunanandaa et al. (2005), who reported no significant difference between controls and the population of transgenic events in *Arabidopsis* transformed with HPPD or HPT under the control of the CaMV 35S promoter. Several studies have indicated that tissue-specific expression of vitamin E pathway genes has a greater impact on enhancing tocopherol production than constitutive over-expression (Savidge et al., 2002; Collakova and DellaPenna, 2003; Karunanandaa et al., 2005).

In potato tubers, the highest increase in tocopherols in our transgenic lines was 2.6-fold, which is lower than increases known to result from environmental stress such as cold (four-fold) (Spychalla and Desborough, 1990). Similarly, in *Arabidopsis* leaves, transgenic approaches have attained a maximum increase in tocopherols of only seven-fold (Kanwischer et al., 2005). Increases of total tocochromanols up to 11.5-fold have been achieved in soybean seed, but were due entirely to tocotrienol accumulation with a net decrease in tocopherols (Karunanandaa et al., 2005). When one considers that under abiotic stress conditions, tocopherol levels increase approximately 18-fold in *Arabidopsis* leaves (Collakova and DellaPenna, 2003a), it is clear that the potential for tocopherol accumulation far exceeds what has been engineered thus far. Understanding induction of tocopherol biosynthesis during stress will be useful for developing strategies to engineer crops with enhanced tocopherol levels.

The results from recent applied research studies to increase vitamin E levels in crop plants leave many open questions on the fundamental aspects of the biochemistry of vitamin E synthesis. Current knowledge cannot explain the accumulation of tocotrienols that occurs when HPPD is expressed in conjunction with the bifunctional prephenate dehydrogenases from *Erwinia herbicola* or *Saccharomyces cerevisiae* (TyrA and Tyr1) (Rippert et al., 2004; Karunanandaa et al., 2005). Equally confounding is the fact that upregulation of GGDR in plants over-expressing HPT, HPPD, and *Erwinia* PDH/CM does not divert flux to tocopherols, in contrast to the result of over-expression of GGDR in *Synechocystis* (Karunanandaa et al., 2005). Furthermore, a possible role for tocopherol cyclase (VTE1) in determining tocopherol composition is emerging through mutant studies (Kanwischer et al., 2005). It is currently unknown how tocopherol synthesis is induced during stress. Our results in potato raise additional questions regarding the regulation of vitamin E synthesis in different plant tissues. A more complete understanding of the regulatory networks coordinating the metabolism of tocopherols and related isoprenoids and the induction of tocopherol synthesis during stress will greatly aid efforts to engineer crops for human health benefits.

Acknowledgements The author gratefully acknowledges the generous contributions of Dr. Dean DellaPenna to this work, through the gift of the genes At-HPPD and At-HPT, by providing access to HPLC equipment, and for indispensable guidance.

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