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Increase of the tocochromanol content in transgenic *Brassica napus* seeds by overexpression of key enzymes involved in prenylquinone biosynthesis

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Abstract Lipid soluble tocochromanols, only synthesised in photosynthetic organisms, are industrially interesting compounds because of their antioxidative properties and their essential function in nutrition. In order to increase the tocochromanol content in the seed oil of transgenic plants, approaches were undertaken to engineer the flux of substrates and intermediates through the pathway. To this end, we overexpressed genes encoding hydroxyphenylpyruvate dioxygenases, alone or in combination with chimeric homogentisate phytyltransferase and tocopherol cyclase genes, in seeds of transgenic *Brassica napus* plants and analysed total tocochromanol content and composition. Overexpression of chimeric hydroxyphenylpyruvate dioxygenase genes, both in the cytosol or in the plastids of developing seeds, yielded a slight although significant increase in total tocochromanol level. Coexpression of a hydroxyphenylpyruvate dioxygenase gene with both a homogentisate phytyltransferase gene and a tocopherol cyclase gene elevated this effect with maximum increases of up to two-fold in individual lines and this phenotype was found to be stably inherited. These data showed that the three enzymes are critical in determining the total tocochromanol content in the seed oil of *Brassica napus* plants, while the tocopherol cyclase, unlike hydroxyphenylpyruvate dioxygenase and homogentisate phytyltransferase, had additionally an effect on the relative abundance of individual tocochromanol species and resulted in an increase of δ-tocopherol and plastochromanol-8 in the seeds.

Keywords Tocochromanol · 4-Hydroxyphenylpyruvate dioxygenase · Homogentisate phytyltransferase · Tocopherol cyclase · *Brassica napus* · Transgenic rapeseed

Abbreviations

DMGGBQ	2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinol
DMPBQ	2,3-dimethyl-5-phytyl-1,4-benzoquinol
DMSBQ	2,3-dimethyl-5-solanesyl-1,4-benzoquinol
HPPD	4-hydroxyphenylpyruvate dioxygenase

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HGGT	homogentisate geranylgeranyl-transferase
HPT	homogentisate phytyltransferase
HST	homogentisate solanesyl transferase
MPBQ	2-methyl-6-phytyl-1,4-benzoquinol
MGGBQ	2-methyl-6-geranylgeranyl-1,4-benzoquinol
MSBQ	2-methyl-6-solanesyl-1,4-benzoquinol
MT1	MPBQ/MGGBQ/MSBQ methyl-transferase
MT2	γ-tocopherol methyltransferase
P-8	plastochromanol-8
TC	tocopherol cyclase

Introduction

The lipid soluble tocochromanols comprise a group of eight related isoforms (Schneider 2005; Traber and Sies 1996). The tocopherols consist of a polar chromanol ring with a saturated C_{16} prenyl side chain and comprise four homologous forms, namely α-, β-, γ- and δ-tocopherol, differing only in the number and position of methyl substituents on the chromanol head group, whereas the tocotrienols possess an unsaturated C_{16} prenyl group but otherwise the same substitution pattern on the chromanol ring. These different tocochromanol forms have varying antioxidative abilities in scavenging oxygen radicals and quenching singlet oxygen, with the α-forms having the highest biological activity (Schneider 2005). In addition tocochromanols appear to play a role in the preservation of membrane integrity by forming complexes with products of membrane lipid hydrolysis as well as in the regulation of transcription and post-translational processes (Azzi et al. 2002; Chan et al. 2001; Munné-Bosch and Falk 2004; Quinn 2004; Yamauchi et al. 2001).

The main features of the biosynthetic pathway of prenylquinones in plants have been elucidated several years ago using classical biochemical methods (Threlfall and Whistance 1971), but the genes encoding the respective enzymes of this pathway have only been cloned during the past few years (Collakova and DellaPenna 2001; Porfirova et al. 2002; Savidge et al. 2002; Schledz et al. 2001; Shintani and DellaPenna 1998). In plants the biosynthesis of the different prenylquinones as well as plastoquinone and, in the seeds of certain plant species, also plastochromanol-8 (P-8) proceeds in plastids (Lichtenthaler et al. 1981; Soll et al. 1985). As shown in Fig. 1, different prenyl diphosphates synthesised via the plastidial 1-deoxyxylulose-5-phosphate (DXP) pathway form the hydrophobic tails (Lichtenthaler 1998; Rodriguez-Concepcion and Boronat 2002; Rohmer 1998), whereas homogentisate provides the basal aromatic ring structure of the polar headgroups (Dahnhardt et al. 2002; Garcia et al. 1999). The 4-hydroxyphenylpyruvate dioxygenase (HPPD) catalyses the conversion of the substrate 4-hydroxyphenylpyruvate to homogentisate in a reaction involving decarboxylation, oxidation and side chain rearrangement (Borowski et al. 2004). The first committed step in the biosynthesis of tocochromanols is catalysed by homogentisate prenyltransferases (HPT, HGGT and HST) which differ in their prenylPP specificities so that the respective 2-methyl-6-prenyl-1,4-benzoquinol intermediates are produced (Collakova and DellaPenna 2001; Hofius and Sonnewald 2003; Sadre et al. 2003; Sattler et al. 2004; Savidge et al. 2002; Soll 1987). Introduction of another methyl group yields 2,3-dimethyl-5-prenyl-1,4-benzoquinols (Marshall et al. 1985). The cyclisation reaction is catalysed by tocopherol cyclase (TC). This enzyme forms the chromanol ring structure of the tocochromanols, which is essential for the antioxidative radical scavenging function, by generating an additional oxygen heterocycle next to the aromatic ring originating from homogentisate (Fig. 1) (Arango and Heise 1998; Soll et al. 1985). In plants the dimethylated intermediates are preferentially cyclised to the respective γ-tocochromanols and further methylated to α-tocochromanols, whereas the cyclisation products derived from the monomethylated prenylquinone intermediates, δ-tocochromanols and the respective methylated β-tocochromanols, are only existent in marginal amounts (Arango and Heise 1998; Porfirova et al. 2002).

HPPD proteins and genes have been isolated from a variety of species including bacteria

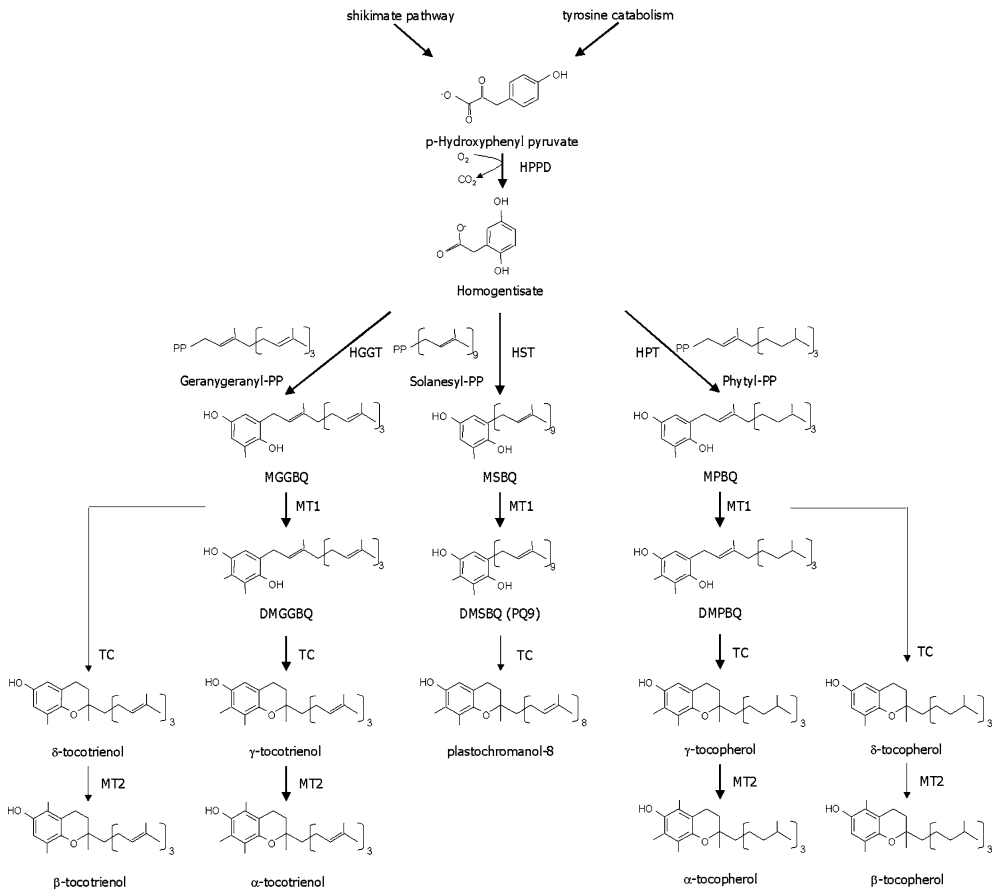

Fig. 1 Schematic representation of prenylquinone biosynthesis in plants. HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; HGGT, homogentisate geranylgeranyltransferase; HST, homogentisate solanesyltransferase; MGGBQ, 2-methyl-6-geranylgeranyl-1,4-benzoquinol; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; MT1, MGGBQ/MPBQ/MSBQ methyltransferase; DMGGBQ, 2,3-dimethyl-5-geranylgeranyl-1, 4-benzoquinol; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinol; DMSBQ, 2,3-dimethyl-5-solanesyl-1,4-benzoquinol; TC, tocopherol cyclase; MT2, γ-tocopherol methyltransferase

(Denoya et al. 1994; Fuqua et al. 1991; Ruetschi et al. 1992), protozoans (Hummel et al. 1992), fungi (Wyckoff et al. 1995), plants (Fritze et al. 2004; Garcia et al. 1999; Kleber-Janke and Krupinska 1997) and mammals (Ruetschi et al. 1997). HPPDs are soluble proteins which form homodimers in eukaryots and homotetramers in prokaryotes (Fritze et al. 2004). They bind one ferrous ion per protein molecule in the active form like all other α-keto-acid-dependent dioxygenases (Borowski et al. 2004). Unlike the HPPDs from mammals and bacteria, the HPPD from plants is involved in the plastidial tocopherol and plastoquinone biosynthesis. Evidence has been provided that in plants HPPD activity is restricted to the cytosol and contributes to both degradation of amino acids and prenylquinone synthesis (Fritze et al. 2004; Garcia et al. 1999; Lenne et al. 1995). The genetic and biochemical interest in this enzyme is due to the fact that the inhibition of HPPD is a new mode of herbicidal action (Borowski et al. 2004; Matringe et al. 2005) and that the HPPD is involved in the regulation of the tocochromanol content in plants (Falk et al. 2003; Kleber-Janke and Krupinska 1997; Tsegaye et al. 2002). So far, HPPD sequences from *Arabidopsis* and barley have been used to investigate the importance of this enzymic activity in controlling tocopherol biosynthetic rates by overexpressing the respective enzymes in

the cytosol of transgenic *Arabidopsis* (Tsegaye et al. 2002) and tobacco plants (Falk et al. 2003).

In this paper we report the overexpression of chimeric HPPD genes from a pro- and an eukaryotic origin under the control of seed specific promoters in transgenic rapeseed plants and elucidate the influence due to different subcellular localisations of the recombinant HPPD enzymes in the cytosol and in the plastids of transgenic *B. napus* embryos on the total tocochromanol content. In addition, we overexpressed chimeric HPPD genes together with HPT and TC genes in the seeds of transgenic plants and analysed their combined impact on tocochromanol composition and content in the seed oil.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli strains XLI blue and DH5α were used for cloning and propagation of recombinant plasmids. *E. coli* TOP10 cells (Invitrogen) were used for expression of recombinant proteins. Transformed cells (Sambrook et al. 1989) carrying expression plasmids were grown at 37°C in LB medium supplemented with 50 μg ml^{-1} kanamycin to an OD_{600} of 0.5. Subsequently expression of the recombinant proteins was induced by addition of arabinose to a final concentration of 0.2% and incubation for 21 h at 37°C.

Development of HPPD expression vectors

Genomic DNA from prokaryotic cells was extracted according to Rippka (1988). Total RNA was extracted from eukaryotic tissue using a LiCl–RNA isolation method (Menhaj et al. 1999). Oligo-dT-Dynabeads (Dynal Biotech GmbH, Hamburg, Germany) were used for mRNA isolation and first strand cDNA synthesis was carried out with MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturers instructions. The open reading frames corresponding to the prokaryotic HPPD gene from *Synechocystis* PCC 6803 (*slr0090*, HPPD1) and the eukaryotic HPPD gene from *Homo sapiens* (X72389, HPPD2) were amplified from genomic DNA (HPPD1) and cDNA (HPPD2) by PCR with *Pfu* polymerase (Promega, Madison, WI, USA) using the specific primer pairs HPPD1-a (ATG GAA TTC GAC TAT CTT CAT TTA TAC GTT G) and HPPD1-b (TTA TGG CAC TTC TAA CTG TTT TTC TAA AGT C) as well as HPPD2-a (ATG ACG ACT TAC AGT GAC AAA GG) and HPPD2-b (TTA CAT CCC GGG CAC CAC CC). The resulting PCR products were ligated into the expression vector pBAD-TOPO (Invitrogen GmBH, Karlsruhe, Germany) according to the manufacturers manual. For expression studies in *E. coli* the corresponding ORFs were amplified by PCR with *Pfu* polymerase using the specific primer pairs HPPD1-*Sph*I (CGC ATG CAA TTC GAC TAT CTT CAT TTA TAC GTT GAC G) and HPPD1-*Bam*HI (CGG ATC CTG GCA CTT CTA ACT GTT TTT CTA AAG TCT CC) as well as HPPD2-*Nco*I (ACC ATG GCG ACT TAC AGT GAC AAA GG) and HPPD2-*Bam*HI (TGG ATC CCA TCC CGG GCA CCA CCC CAT TGG). The PCR products were phosphorylated with T4-DNA polymerase (MBI Fermentas, St. Leon-Rot,Germany) subcloned into pUC19/*Sma*I and finally ligated into the *Sph*I/*Bam*HI and *Nco*I/*Bam*HI sites of the expression vectors pQE70 and pQE60 (Qiagen, Hilden, Germany), respectively, so that the His-tag sequence of the respective vector was added in frame to the 3′ end of the open reading frame.

For construction of the plant expression vectors, expression cassettes with the coding regions of HPPD1 and HPPD2 under the control of the seed specific DC3Ω promoter sequence from *Daucus carota* with the Ω-enhancer sequence from *Nicotiana tabacum* or the napin promoter from *Brassica napus* and the nopaline synthase termination region (nos terminator) from *Agrobacterium tumefaciens* were developed and integrated into the pPZP111 plant transformation vector (Hajdukiewicz et al. 1994). For plastidial targeting, the transit peptide (Tp) sequence of the RUBISCO small subunit (SSU) from potato was fused to the HPPD1 and HPPD2 ORF, respectively. For the construction of pDC3-HPPD1, the DC3Ω sequence was amplified by PCR with *Pfu* polymerase using the primers DC3Ω-*Sbf*I (AAC CTG CAG GCC GAG CTA ACC ACA ACT C)

and DC3Ω-*Eco*RI (GGG AAT TCC ATC CAT GGT AAT TGT AAA TGT AAT) introducing a *Sbf*1 and an *Eco*RI restriction site at the 5′ and 3′ end, respectively. The nos terminator sequence was amplified using Nos-*Xho*I (GCT CGA GAG TCA AGC AGA TCG TTC AAA CAT TTG G) and Nos-*Sbf*I (GCC TGC AGG TCG ATC TAG TAA CAT AGA TGA C). For the amplification of the HPPD1 ORF the primer pair HPPD1-*Mlu*I/*Eco*RI (AAC GCG TTA GGT GCA TGG AAT TCG ACT ATC TTC) and HPPD1-*Xho*I (GCT CGA GTT ATG GCA CTT CTA ACT G) was used. Blunt end PCR products were phosphorylated with T4-DNA polymerase (New England Biolabs, Beverly, MA, USA) and subcloned in a pBluescript II KS+ vector (pBSII) restricted with *Sma*I and *Eco*RV to remove the *Eco*RI restriction site within the multiple cloning site. pBSII containing the HPPD1 PCR product (pHPPD1) was cut with *Bsp120*I and, after Klenow fill in, restricted with *Xho*I. The nos regulatory sequence was digested with *Hind*II and *Xho*I and inserted into the *Bsp*120I/Klenow-*Xho*I site. The DC3Ω promoter sequence was subsequently cloned into the *Bam*HI and *Eco*RI restriction sites. The expression cassette was then inserted into the *Sbf*I site of pPZP111 yielding the plant transformation construct pDC3-HPPD1. In order to fuse the plastidial targeting sequence of the RUBISCO small subunit to the HPPD1, the DC3Ω and the targeting sequence were amplified with the following primers DC3Ω-*Sbf*I (AAC CTG CAG GCC GAG CTA ACC ACA ACT C), DC3Ω-*Nco*I (GGC CAT GGT AAT TGT AAA TGT AAT TGT AAT) and SSU-*Nco*I (GGC CAT GGC TTC CTC TGT TAT TT), SSU-*Mlu*I (CTA ACG CGT CCA CCA TTG). The amplified SSU fragment was cloned into the vector pBSII and the *Mlu*I/*Xho*I excised HPPD1 fragment from pHPPD1 was cloned in frame to the SSU transit peptide sequence (Tp). The resulting vector was cut with *Bsp*120I, blunted with Klenow and digested with *Xho*I. The *Hind*II/*Xho*I digested nos terminator sequence was inserted. After partial *Nco*I restriction, the DC3Ω-promoter was cloned upstream of the chimeric TpHPPD1 ORF into the *Bam*HI/*Nco*I site. Finally, the whole expression cassette was excised with *Sbf*I and inserted into pPZP111 giving pDC3-TpHPPD1 (Fig. 3). For the construction of pDC3-HPPD2 and pDC3-TpHPPD2 a *Nco*I and a *Xho*I restriction site or a *Mlu*I and a *Xho*I site were added at the 5′ and 3′ end of the HPPD2 sequence via PCR using the primers HPPD2-*Nco*I (GCC ATG GCG ACT TAC AGT GAC AAA GGG) or HPPD2-*Mlu*I (GAC GCG TTA GGT GCA TGA CGA CTT ACA GTG ACA AAG GG) and HPPD2-*Xho*I (GCT CGA GTT ACA TCC CGG GCA CCA CCC CAT TGG), respectively. The *Nco*I/*Xho*I and the *Mlu*I/*Xho*I HPPD2 fragments were exchanged against the HPPD1 and TpHPPD1 sequences in the respective expression cassettes and after *Sbf*I restriction inserted into pPZP111 yielding pDC3-HPPD2 and pDC3-TpHPPD2 (Fig. 3). pNap-HPPD2 and pNap-TpHPPD2 were created using the primers Napin-*Smi*I-*Pme*I-*Hind*III (CAT TTA AAT GTT TAA ACA AGC TTA TTT CTC TTT TCG) and Napin-*Nco*I (GCC ATG GGT GTA TGT TTT GTA GTG ATG AGT TTT GG) for the amplification of the promoter. The napin sequence was cloned into the *Sma*I site of pUC19 and the nos terminator was amplified using primers nos-*Not*I-*Xho*I (GGC GGC CGC CTC GAG AGT CAA GCA GAT CGT TCA AAC ATT TGG) and nos-*Pme*I-*Smi*I (CAT TTA AAT GTT TAA ACT CGA TCT AGT AAC ATA GAT GAC ACC G) and cloned into pUC19/*Sma*I. The nos terminator was excised with *Ecl*136II/*Hind*II and inserted into the *Hind*II site of the promoter-containing vector. The napin promoter-nos terminator cassette was cut with *Nco*I/*Xho*I and the HPPD2 sequences with or without Tp sequence were cut with *Nco*I/*Xho*I and cloned into the cassette resulting in pNap-HPPD2 and pNap-TpHPPD2 (Fig. 3), respectively.

Development of HPT-HPPD and HPT-HPPD-TC expression vectors

For construction of the chimeric double gene vectors for plant transformation, an expression cassette with the coding region of HPT (AY089963) from *Arabidopsis thaliana* under the control of the seed specific napin promoter from *Brassica napus* and the nopaline synthase termination region (nos terminator) from *Agrobacterium tumefaciens* was developed and, together

with the expression cassettes from pDC3-HPPD2 and pDC3-TpHPPD2, integrated into the pPZP111 plant transformation vector (Hajdukiewicz et al. 1994).

For the construction of the HPT expression cassette the napin promoter and the nos terminator were amplified by PCR using the primers Napin-*Smi*I-*Pme*I-*Hind*III (CAT TTA AAT GTT TAA ACA AGC TTA TTT CTC TTT TCG) and Napin-*Sal*I (TTG GTC GAC TTT TGT AGT GAT GAG TTT TGG), as well as nos-*Not*I-*Xho*I (GGC GGC CGC CTC GAG AGT CAA GCA GAT CGT TCA AAC ATT TGG) and nos-*Pme*I-*Smi*I (CAT TTA AAT GTT TAA ACT CGA TCT AGT AAC ATA GAT GAC ACC G) in order to introduce suitable restriction sites. The resulting PCR products were cloned into pUC19/*Sma*I, yielding pUC19-Napin and pUC19-nos, respectively. The pUC19-Napin was then cut with *Sal*I and religated in order to remove the *Sal*I-site from the multiple cloning site. The nos terminator sequence was excised from pUC19-nos by *Ecl*136II/*Hind*II restriction and ligated into the *Pst*I cut and T4 DNA Polymerase blunted pUC19-Napin resulting in the pUC19-Napin-nos. The primers HPT-*Sal*I (CGT CGA CCA AAT GGA GTC TCT GCT CTC TAG TTC TTC TC) and HPT-*Not*I (GGC GGC CGC TCA CTT CAA AAA AGG TAA CAG C) were used for the amplification of the *Arabidopsis thaliana* HPT using cDNA synthesised from isolated leaf mRNA as template, as described above. The *Sal*I/*Not*I cut PCR fragment was ligated into the *Sal*I/*Not*I sites of pUC19-Napin-nos and the complete HPT expression cassette was excised with *Hind*III and cloned into pPZP111/*Hind*III, giving pHPT. Subsequently, the DC3-HPPD2 and DC3-TpHPPD2 expression cassettes were excised from the respective vectors with *Sbf*I and inserted into *Sbf*I linearised pHPT yielding pHPT-HPPD2, pHPT-TpHPPD2 and –2a (Fig. 5). pHPT-TpHPPD2 and –2a, with unidirectional and divergent orientation of the expression cassettes, were identified by restriction analysis.

For the construction of the chimeric triple gene constructs pHPT-TpHPPD2-ZmTC and pHPT-TpHPPD2-AtTC (Fig. 5) an additional ZmTC and AtTC expression cassette (Kumar et al. 2005) was integrated into the *Smi*I-site of pHPT-HPPD2a.

Functional expression in *E. coli*

TOP10 cells containing the respective pBAD expression vector or XL1-Blue cells harbouring the repressor plasmid pREP4 (Qiagen, Hilden, Germany) and the respective pQE expression vector were suspended in 0.1 M sodium phosphate buffer, 2 mM PMSF, pH 7.2 and disrupted by sonication. Soluble protein fractions obtained by high-speed centrifugation were used for Western blot analysis and enzymatic assays.

Protein concentration was estimated by Bradford protein assays using bovine serum albumin as standard. Standard methods were used for SDS-PAGE analysis (Laemmli 1970). For Western blot analysis, proteins were transferred to PVDF membranes by semi-dry blotting and the His-tagged recombinant HPPD proteins were detected by chemiluminescence (LAS-1000, Raytest, Straubenhardt, Germany) using penta-His antibodies from mice (Qiagen) and goat anti-mouse IgG-POD conjugate antibodies (Qiagen) with Lumi-Light plus (Roche, Mannheim, Germany) as substrate. Pre-stained and His-tagged (Best of all) protein markers were obtained from New England Biolabs (Beverly, MA, USA) and Biomol (Plymouth Meeting, PA, USA), respectively.

For enzymatic assays (Schulz et al. 1993) the labelled substrate [U-^{14}C]hydroxyphenylpyruvate was synthesised from 0.1 μCi [U-^{14}C]tyrosine with 0.025 U L-amino acid oxidase (Sigma) in the presence of 30 U catalase (Sigma) in a volume of 100 μl 0.1 M sodium phosphate buffer pH 7.2. After 30 min incubation at 37°C 100 μl 0.1 M sodium ascorbate pH 7.0 were added and the dioxygenase reaction was started by the addition of *E. coli* protein extracts. After 30 min at 37°C the reaction was stopped by adding 100 μl 2.5 M HCl. The reaction product homogentisate was extracted with 1 ml ethyl acetate, dried under N_2 and redissolved in 100 μl ethyl acetate, triggered with 50 μg unlabelled homogentisic acid. The extract was separated by TLC in a toluol:isoamyl alcohol:acetic acid (40:20:1.5) mobile phase and

the homogentisic acid was quantified via scintillation counting.

Development and analysis of transgenic *Brassica napus* plants

Hypocotyl segments of the spring rapeseed cultivar 'Drakkar' were transformed with *A. tumefaciens* C58C1 ATHV cells harbouring the respective chimeric plant expression construct and regenerated to intact plants as described earlier (Zarhloul et al. 1999). Successful gene transfer was initially confirmed by NPTII ELISA assays (Agdia Inc., Elkhart, IN, USA) with leaf extracts of T1 plantlets. Transgenic plants were compared with regard to their seed yield, their seed oil content as well as their total tocochromanol content in the seed oil. Transgenic plants meeting these criteria best were selected and used as parental lines for growth of the next generation. Transgenic plants of the T1, T2 and T3 generation were grown under temporally and spatially different environments (average temperature 15°C, 16 h daylight) in the greenhouse in order to increase seed material and to confirm the transgenic phenotype in the seed progeny in comparison to respective control plants (cv. Drakkar).

Following the extraction of T2, T3 or T4 seeds (0.3 g pooled seeds) with petroleum ether, tocopherols were analysed by normal-phase HPLC with fluorescence detection (295 nm excitation, 320 nm emission). The different tocochromanols were identified due to their retention times referred to commercially available standards manufactured by Matreya (Pleasant Gap, PA, USA) and purchased from Biotrend (Cologne, Germany) or obtained from Calbiochem (Merck Biosciences GmbH, Bad Soden, Germany), while plastochromanol-8 (P-8) was identified by cochromatography of seed oil from *Linum usitatissimum*, which is known to contain high levels of up to 20% P-8 in its seed oil (Balz et al. 1992). The total tocochromanol content in the seed oil (mg kg^{-1} oil) of wild-type and transgenic *Brassica napus* plants was calculated as the sum of α-, γ- and δ-tocopherol as well as P-8, with β-tocopherol and 5,7-dimethyltocol as internal standards and iso-octane as sample solvent (Olejnik et al. 1997; Thies 1997).

Statistical analysis of variance (ANOVA) and multiple comparisons of means were performed using SPSS version 10. Box and whisker have been used to calculate maximum, minimum, median, upper quartile, lower quartile and outliers. They form a diagram, which provides a simple graphical summary of a set of data. Outliers are data points out of the outer fences. They are marked with the sign (* or o).

Results and discussion

Cloning and functional characterisation of HPPD genes

Utilising the sequence informations available in the databases [http://www.kazusa.or.jp/cyano; http://www.ncbi.nlm.nih.gov/entrez] the open reading frames of the HPPD gene from *Synechocystis* PCC6803 and the HPPD gene from *Homo sapiens* were cloned via PCR from genomic DNA and cDNA, respectively. The encoded prokaryotic (HPPD1) and eukaryotic (HPPD2) proteins with a mass of about 38.5 and 45 kDa, respectively, were both expressed in *E. coli* and were found to behave like soluble proteins (Fig. 2). The expression level of the recombinant HPPDs in *E. coli* was analysed by SDS-PAGE and Western blot. As shown in Fig. 2A, both HPPD1 and HPPD2 accumulated in approximately the same amounts in the soluble fractions of *E. coli*. *In vitro* assays conducted with these

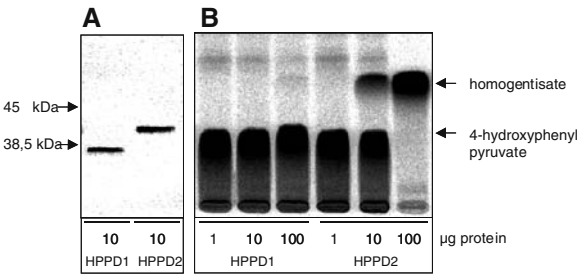

Fig. 2 (**A**) Western blot analysis of soluble fractions from *E. coli* cells expressing the recombinant His-tagged HPPD1 and HPPD2. (**B**) HPPD activity in *E. coli* cells overexpressing the recombinant HPPD1 and HPPD2 protein. Separation of the substrate [U-^{14}C]*p*-hydroxyphenylpyruvate and the reaction product [U-^{14}C]homogentisate of the dioxygenase assays was carried out via TLC as described in material and methods

soluble fractions of *E. coli* cells and [U-^{14}C]*p*-hydroxyphenylpyruvate as substrate, however, revealed that the eukaryotic enzyme converted the substrate [U-^{14}C]*p*-hydroxyphenylpyruvate to homogentisate more effectively than the prokaryotic one (Fig. 2B). At a protein concentration of 100 μg only marginal product formation was observed with the *E. coli* soluble fractions containing HPPD1, whereas the respective HPPD2 fractions catalysed a complete turnover of the substrate, which corresponds to a 100-fold higher activity of the eukaryotic HPPD under these conditions.

Overexpression of chimeric HPPD gene constructs in transgenic *B. napus* plants

In order to elucidate the relevance of the HPPD within the course of the tocochromanol biosynthesis and its impact on tocochromanol quantity, we produced transgenic *B. napus* plants overexpressing different chimeric HPPD1 genes. For this purpose we developed binary plant transformation vectors harbouring the *Npt*II gene as selection marker and a chimeric HPPD gene, in

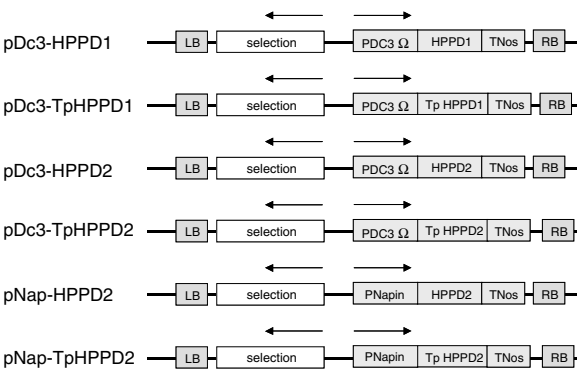

Fig. 3 Schematic representation of T-DNA harbouring the chimeric gene constructs for plant transformation containing the prokaryotic HPPD1 and the eukaryotic HPPD2 in the absence or presence of a plastidial targeting sequence (Tp) under the control of two different seed specific promoters. LB, left T-DNA border; selection, neomycin phosphotransferase sequence under the control of a cauliflower mosaic virus 35S promoter and termination sequence; PNapin, seed specific napin promoter from *B. napus*; PDC3 Ω; seed specific DC3 promoter from *Daucus carota* with Ω-enhancer sequence from *Nicotiana tabacum*; TNos, transcription termination sequence of the nopaline synthase gene from *A. tumefaciens*; RB, right T-DNA border

which we placed the HPPD1 and HPPD2 coding sequence either under the control of the weak seed specific DC3Ω (Spiekermann 2005) or the strong seed specific napin promoter, and fused the sequence of the RUBISCO small subunit transit peptide (Tp) in frame to the respective HPPD sequences (Fig. 3). Transgenic *Brassica napus* lines were generated by *Agrobacterium tumefaciens* mediated transformation. Via HPLC we analysed NPTII-ELISA positive transgenic *B. napus* plants and their progeny with regard to tocochromanol composition and content.

The tocochromanol content of transgenic *B. napus* T2 seeds expressing one of the different constructs are shown in Fig. 4 in comparison to the respective data of control plants. A cultivation of the three groups of plants under different conditions caused variations in the tocochromanol content (Marwede et al. 2004) as reflected by the data of the control plants. In comparison to the control plants, the average total tocochromanol content in the seed oil of the T1 populations harbouring DC3-HPPD1 or DC3-HPPD2 was increased by 6% and 15%, respectively. Hence, similar to the results obtained by the heterologous expression experiments in *E. coli* (Fig. 2B), the expression of the eukaryotic HPPD2 in the seeds of transgenic *B. napus* plants caused a higher increase in the average total tocopherol content of the seed oil compared to the prokaryotic HPPD1, but *in planta* the difference between the pro- and eukaryotic HPPD was not as pronounced as in *E. coli*. The effect of different regulatory sequences on HPPD2 expression and total tocochromanol content in the seed oil of transgenic *B. napus* plants is also displayed in Fig. 4. Surprisingly, the expression of HPPD2 under the control of the weak DC3Ω-promoter resulted in higher overall tocochromanol levels in the seeds of T1 plants than in those plants expressing the HPPD2 under the control of the strong napin promoter.

The analyses of T3 seeds from selected plants of the T2 population grown in the same environment verified the enhancement in total tocochromanol content both on an average and on an individual plant level. The difference in the average increase in the total tocochromanol content between DC3-HPPD1 on the one hand and

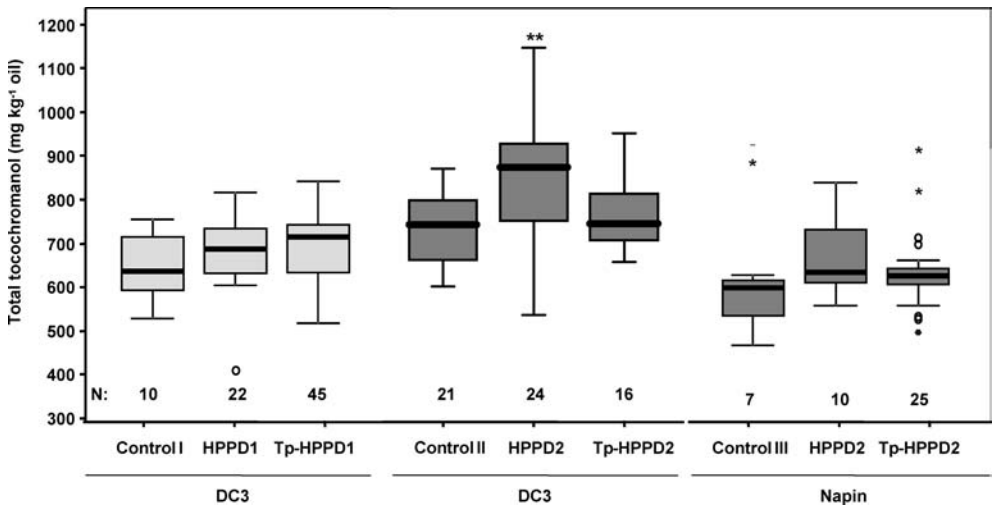

Fig. 4 Tocochromanol content of transgenic *Brassica napus* T2 seeds from independent transgenic T1 lines expressing HPPD1, Tp-HPPD1, HPPD2 and Tp-HPPD2 under the control of the seed specific DC3Ω promoter as well as HPPD2 and Tp-HPPD2 under the control of the napin promoter. T1 plants were grown in different environments, hence the absolute values for the respective wild-type plants differed. * or °, outliers, data points out of the outer fences; N, number of analysed wild type and independent transgenic plants. # significant with $p < 0.05$

DC3-HPPD2 and Nap-HPPD2 on the other hand, was less pronounced (Table 1). The total tocochromanol content in the T3 seeds from selected plants of the T2 population expressing the pro- or eukaryotic HPPD (DC3-TpHPPD1 and Nap-TpHPPD2) amounted to about 1000 mg kg^{-1} seed oil corresponding to an increase of approximately 50% compared to the average control (Table 1) mainly due to a higher α- and γ-tocopherol level. The differences in the α- und γ-tocopherol composition probably derive from variations in γ-tocopherol methyltransferase activity of the different transformants (Table 1). Our data of the transgenic *B. napus* plants overexpressing HPPD1 or HPPD2 is in good accordance with those of Tsegaye et al. (2002) and of Falk et al. (2003), obtained via overexpression of plant HPPDs in the seeds of *Arabidopsis* and tobacco plants, respectively. Regardless of the construct and the plant species significant but only moderate increases in total tocochromanol content could be obtained.

In order to analyse whether the relatively low increase in the total tocochromanol content of the HPPD overexpressing plants was primarily caused by homogentisate degradation in the cytosol, the recombinant HPPDs were targeted to the chloroplasts in the seeds of transgenic *B. napus* plants by fusion to a transit peptide. As shown in Fig. 4, the total tocochromanol content in the seed oil of transgenic T1 plants harbouring DC3-TpHPPD1 was slightly higher than the tocochromanol content of the respective DC3-HPPD1 plants, which express the enzyme in the cytosol. The plastidial expression of the eukaryotic HPPD2 under the control of the DC3 as well as the napin promoter resulted in lower tocochromanol levels than the respective cytosolic expression. In the T2 populations and especially in selected individual T2 plants, however, the differences were less pronounced and results comparable to those achieved by cytosolic HPPD expression were obtained. Maximal increases in the total tocochromanol level of about 20% in the average (DC3-TpHPPD1) and 50% in the best individual plants (Nap-TpHPPD2) producing homogentisate in the plastids were measured (Table 1).

The fact that both a cytosolic and a plastidial HPPD expression result in a slight but significant increase in the total tocochromanol content of transgenic plants indicates that the tocochromanol content is primarily limited neither by a degradation of homogentisate in the cytosol nor by a different accessibility of the cytosolic and plastidial homogentisate pools for tocopherol

Table 1 Tocochromanol composition and content of transgenic *Brassica napus* T3 seeds expressing HPPD1 and HPPD2, respectively

	N	Tocochromanol content (mg kg^{-1} oil)					
		α	γ	δ	P8	Total	Total (%)
T2 population							
Wild-type	52	167 ± 2.7	487 ± 04.8	10 ± 0.2	5.7 ± 0.2	670 ± 06.1	100
DC3-HPPD1	43	252 ± 13*	499 ± 07.8*	9.6 ± 0.4	9.0 ± 0.4*	769 ± 11.9*	115*
DC3-TpHPPD1	35	191 ± 3.4*	584 ± 15.4*	14.3 ± 0.8*	17.1 ± 1.7*	806 ± 18.9*	120*
DC3-HPPD2	31	183 ± 06.0	606 ± 12.3*	16.7 ± 1.1*	13.0 ± 0.9*	819 ± 13.9*	122*
Nap-HPPD2	7	237 ± 15.0	535 ± 35.0	19.3 ± 1.8	13.4 ± 1.7	804 ± 41.0	120
Nap-TpHPPD2	31	183 ± 03.9	519 ± 10.1	17.3 ± 1.3*	10.0 ± 0.7*	729 ± 13.5*	109*
Individual T2 plants							
DC3-HPPD1	–	455	518	10	11	994	148
DC3-TpHPPD1	–	216	767	25	20	1028	153
DC3-HPPD2	–	196	761	23	12	992	148
Nap-HPPD2	–	263	636	21	16	937	140
Nap-TpHPPD2	–	488	511	18	17	1033	154

Mean values ± standard error and individual values of selected plants are shown. Transgenic plants were selected due to their seed yield, seed oil content as well as their total tocochromanol content in the oil of the harvested seeds. (*N*, number of analysed transgenic and control plants). The Nap-HPPD2 population comprised only seven individuals, therefore no statistical evaluation was possible

*$p < 0.05$, significant

synthesis. The data rather suggest that the tocopherol synthesis is limited by the downstream enzyme activities involved in the pathway (Fig. 1), especially the homogentisate phytyltransferase (HPT) and the tocopherol cyclase (TC). Individual overexpression of these two enzymes in the seeds of transgenic *Brassica napus* plants led, similar to the results obtained by the sole overexpression of HPPD, to a significant but moderate rise in the total tocochromanol content, as demonstrated by us and other groups (Collakova and DellaPenna 2003; Kumar et al. 2005; Savidge et al. 2002; Schledz et al. 2001). This increases in the total tocochromanol content in the seed oil of transgenic plants expressing a HPPD, HPT or TC on the one

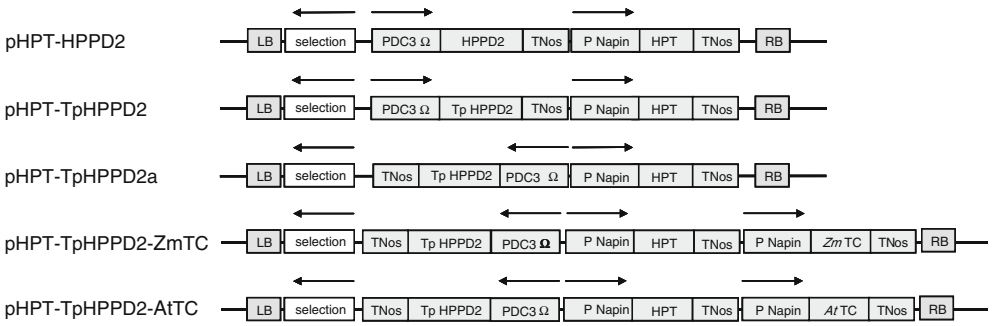

Fig. 5 Schematic representation of T-DNA harbouring the chimeric gene constructs for plant transformation containing the HPT-HPPD and HPT-HPPD-TC expression cassettes. LB, left T-DNA border; selection, neomycin phosphotransferase sequence under the control of a cauliflower mosaic virus 35S promoter and termination sequence; pNapin, seed specific napin promoter from *B. napus*; PDC3Ω; seed specific DC3 promoter from *Daucus carota* with Ω-enhancer sequence from *Nicotiana tabacum*; TNos, transcription termination sequence of the nopaline synthase gene from *A. tumefaciens*; Tp, transit peptide of the RUBISCO small subunit from potato; RB, right T-DNA border

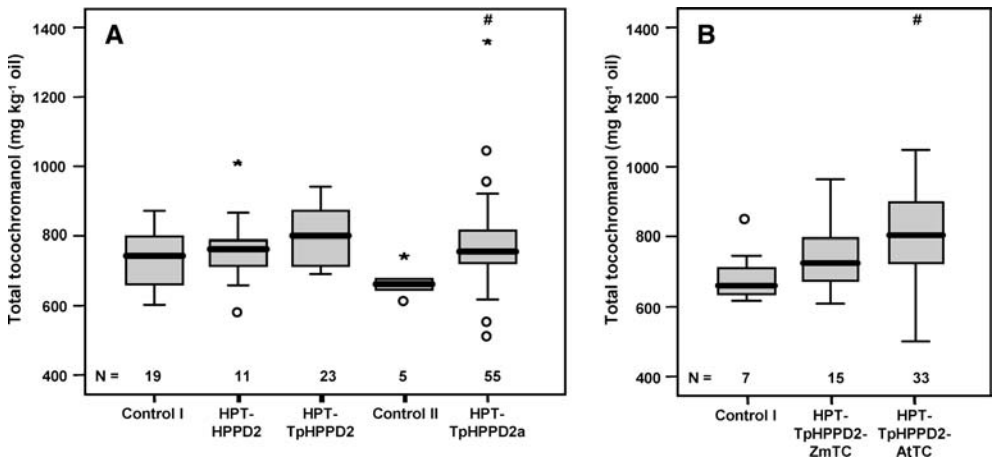

Fig. 6 Tocochromanol content of transgenic *Brassica napus* T2 seeds from independent transgenic T1 lines expressing (**A**) HPT-HPPD- and (**B**) HPT-HPPD-TC constructs. T1 plants were grown in different environments, hence the absolute values for the respective wild-type plants differed. * or °, outliers, data points out of the outer fences; *N*, number of analysed wild type and independent transgenic plants. # significant with $p < 0.05$

hand confirmed a key role of these enzymes in tocopherol biosynthesis, but on the other hand clearly underlined that the metabolic flux in pathways is often not controlled by a single rate limiting enzyme.

In order to elucidate the concerted impact of HPPD, HPT and TC on the tocopherol content in the seed oil of transgenic plants, we transformed different chimeric double and triple gene constructs into rapeseed plants (Fig. 5). The results of the analysis of transgenic T1 plants harbouring one of the different HPT-HPPD2 constructs are displayed in Fig. 6A. Compared to the respective wild-type plants, seed tocochromanol levels of up to 1.2-fold in the average and up to 2-fold in the best-selected transgenic plants could be obtained. Fig. 6B shows the results of the analysis of the T2 seed oil of transgenic T1 plants harbouring HPT-TpHPPD2-ZmTC or HPT-TpHPPD2-AtTC. The constructs differ from each other due to the fact that they contain TC sequences from a monocotyledonous (ZmTC) and dicotyledonous plant (AtTC), respectively (Kumar et al. 2005). Similar to the transgenic plants harbouring a double gene construct average increases in the seed tocochromanol content of about 1.2-fold were determined and the tocochromanol yield in selected transgenic events ranged from 1.4-fold for HPT-TpHPPD2-ZmTC plants to 1.5-fold for HPT-TpHPPD2-AtTC ones. Very similar results were also being obtained for transgenic plants harbouring a triple construct with a further prokaryotic chimeric HPPD gene from *Shewanella oneidensis* (data not shown).

Since the orientation of contiguous genes has often considerable influence on their expression level (Padidam and Cao 2001), we tested the impact of unidirectional and divergent orientations of the HPT and HPPD2 expression cassettes (Fig. 5) on expression and total tocochromanol level in the seed oil of transgenic plants. Our analyses confirmed the relation between transgene orientation and expression level. These T2 seed data were corroborated by the T3 seed oil analyses of the respective transgenic lines (Table 2). Better effects on seed tocochromanol level were achieved with a divergent orientation of the chimeric HPT and TpHPPD2 genes (HPT-TpHPPD2a) than with the unidirectional one (HPT-HPPD2, HPT-TpHPPD2). While the latter yielded a maximal increase of about 1.5-fold, the divergent orientation of the expression cassettes (HPT-TpHPPD2a) resulted in a 1.8-fold enhancement of the total tocochromanol content. This is in accordance to the data shown by Karunanandaa et al. (2005) displaying similar increases of maximal 1.75-fold in the total tocochromanol content of transgenic *Brassica napus* seeds expressing chimeric HPPD-HPT constructs.

Table 2 Tocochromanol composition and content of transgenic *Brassica napus* T3 and T4 seeds expressing HPT-HPPD and HPT-HPPD-TC constructs

	N	Tocochromanol content (mg kg^{-1} oil)					
		α	γ	δ	P8	Total	Total (%)
HPT-HPPD2							
T2 population							
Wild-type	52	167 ± 2.7	487 ± 4.8	10 ± 0.2	5.7 ± 0.2	670 ± 6.1	100
HPT-HPPD2	46	194 ± 6.1*	576 ± 8.3*	15.9 ± 0.6*	8.9 ± 0.5*	795 ± 13.5*	119*
Wild-type	29	311 ± 9.0	417 ± 9.3	9.7 ± 0.2	9.8 ± 0.9	748 ± 12.0	100
HPT-TpHPPD2	36	360 ± 9.3*	495 ± 13.3*	14.1 ± 0.5*	9.4 ± 0.5	879 ± 18.3*	118*
HPT-TpHPPD2a	35	457 ± 15.3*	602 ± 14.5*	24.6 ± 1.3*	12.1 ± 0.7	1096 ± 22.2*	147*
Individual T2 plants							
HPT-HPPD2	–	406	667	28	16	1116	167
HPT-TpHPPD2	–	380	668	20	9	1076	144
HPT-TpHPPD2a	–	618	696	29	20	1363	182
HPT-HPPD-TC							
T2 population							
Wild-type	29	311 ± 9.0	417 ± 9.3	9.7 ± 0.2	9.8 ± 0.9	748 ± 12.0	100
HPT-TpHPPD2- ZmTC	42	322 ± 7.9	578 ± 17.5*	16.8 ± 0.6*	14.3 ± 0.9	931 ± 16.3*	125*
HPT-TpHPPD2-AtTC-I	43	371 ± 12.8*	676 ± 13.0*	74.9 ± 3.8*	29.2 ± 3.3*	1151 ± 19.9*	154*
HPT-TpHPPD2-AtTC-II	46	355 ± 11.8	507 ± 9.9*	43.9 ± 5.1*	26.6 ± 1.8*	933 ± 13.3*	125*
Individual T2 plants							
HPT-TpHPPD2- ZmTC-1	–	422	664	21	12	1118	150
HPT-TpHPPD2- ZmTC-2	–	312	485	23	13	833	111
HPT-TpHPPD2- ZmTC-3	–	387	484	17	28	916	122
HPT-TpHPPD2-AtTC -I1	–	664	669	104	16	1453	194
HPT-TpHPPD2-AtTC -I2	–	424	777	118	14	1322	177
HPT-TpHPPD2-AtTC -I3	–	328	685	139	62	1213	162
HPT-TpHPPD2-AtTC -I4	–	406	622	61	74	1162	155
HPT-TpHPPD2-AtTC -II1	–	609	522	20	69	1220	163
HPT-TpHPPD2-AtTC -II2	–	372	508	171	33	1083	145
Selected T3 plants							
Wild-type	25	349 ± 17.8	401 ± 7.9	11 ± 0.6	11 ± 0.9	772 ± 19.9	100
HPT-TpHPPD2-AtTC-I1	41	388 ± 11.1	712 ± 15.4*	104 ± 3.1*	21 ± 1.9*	1224 ± 21.8*	159
HPT-TpHPPD2-AtTC-I2	46	401 ± 10.3*	698 ± 10.6*	107 ± 2.8*	26 ± 1.2*	1232 ± 16.6*	160
Individual T3 plants							
HPT-TpHPPD2-AtTC-I1	–	610	1010	163	66	1849	240
HPT-TpHPPD2-AtTC-I2	–	670	739	115	47	1570	203

Mean values ± SE and individual values of selected plants are shown. Transgenic plants were selected due to their seed yield, their seed oil content as well as their total tocochromanol content in the oil of the harvested seeds. (*N*, number of analysed transgenic and control plants). T1, T2 and T3 plants were grown in different environments, hence the absolute values for the respective wild-type plants differed

*$p < 0.05$, significant

As shown in Table 2, the improved seed tocochromanol content observed in the seeds of HPPD-HPT-AtTC and HPPD-HPT-ZmTC plants overexpressing not only HPPD and HPT but also a TC sequence was stably inherited as well. In a selected HPT-TpHPPD2-AtTC-I1 plant the seed tocochromanol level increased from 1450 mg kg^{-1} oil to 1850 mg kg^{-1} oil. In comparison to the results obtained with the double gene constructs these data suggest that the coexpression of TC with HPPD and HPT can enhance total seed tocochromanol level.

Moreover, TC overexpression, unlike that of HPT, significantly altered the tocochromanol composition (Fig. 7), as observed in transgenic rapeseed plants expressing a chimeric TC gene only (Kumar et al. 2005). A high cyclase expression, and accordingly, a high cyclase activity

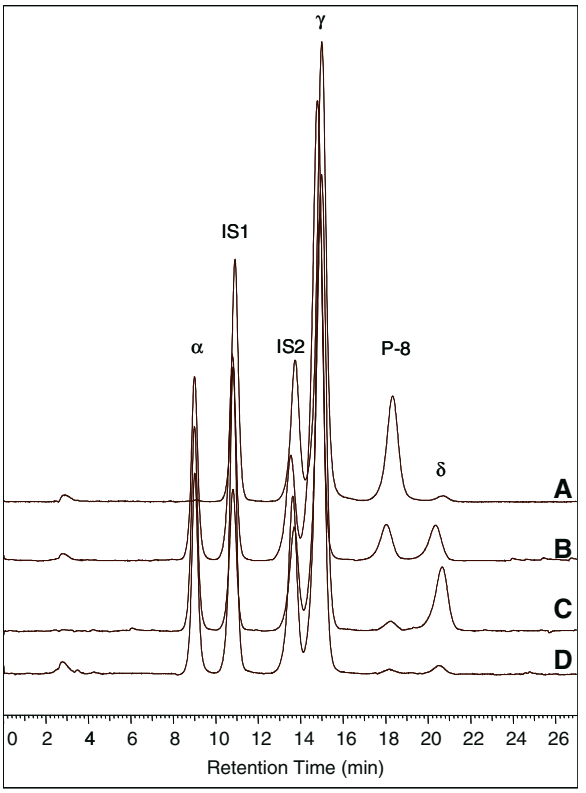

Fig. 7 HPLC analysis of the total tocochromanol content of transgenic *Brassica napus* T4 seeds from individual transgenic T3 plants expressing HPT-HPPD-AtTC constructs in comparison to natural tocochromanol reference mixtures from *Brassica napus* and *Linum usitatissimum*. Total seed lipids were extracted, and the tocochromanols present in the oil were separated by normal-phase HPLC and detected using a fluorescence detector; 290 nm excitation and 325 nm emission (**A**) Natural tocochromanol mixture from *Linum usitatissimum* seed oil. (**B**) and (**C**) Seed oil from transgenic *Brassica napus* plants (T3) harbouring HPT-TpHPPD2-AtTC-I, possessing varying levels of P-8 and δ-tocopherol. (**D**) Seed oil from wild-type *Brassica napus* plants grown under the same environmental conditions as the transgenic plants. IS1, Internal standard 1: 5,7-dimethyltocol; IS2, Internal standard 2: β-tocopherol; Retention times of the different tocochromanols were determined by HPLC analysis of tocopherol standards and natural reference mixtures

enabled the utilisation not only of DMPBQ as a substrate, but also of the other prenylquinone intermediates MPBQ and plastoquinone 9, giving rise to an up to 17-fold higher δ-tocopherol and 7.5-fold higher P-8 level in seeds derived from selected T2 and T3 plants (HPT-TpHPPD2-AtTC-I4 and -II2) as shown in Table 2.

In summary, coexpression of two or three genes essential for tocopherol synthesis in developing seeds of transgenic rapeseed plants resulted in a 2.4-fold increase in the tocopherol content of the seed oil. To achieve a further increase in the tocopherol content, amongst others the plastidial phytylPP pool and the properties of the HPT need to be improved (Falk et al. 2005; Karunanandaa et al. 2005; Sadre et al. in preparation; Valentin and Qi 2005). These assumptions are in line with recent data showing that a boost in the plastidial homogentisate pool in transgenic plants resulted in the activation of an endogenous homogentisate geranylgeranyltransferase (HGGT) activity and, thus, in the accumulation of tocotrienol whereas the tocopherol content was hardly effected (Karunanandaa et al. 2005; Rippert et al. 2004). A concomitant increase in the plastidial phytylPP pool in such transgenic plants as suggested by Valentin and Qi (2005) and Ischebek et al. (2006) will show whether the tocopherol content is primarily controlled by the substrate pools available to the HPT or the properties of enzymes. Altogether the data obtained by the analysis of the tocochromanol biosynthesis point out that a several fold increase of the tocochromanol content in the seeds of transgenic plants requires an upregulation of the pathway by overexpressing multiple rate engineering enzymes as well as a reinforced supply of the aromatic and isoprenoid precursors. Hence, the analyses of these transgenic plants emphasised the complex mechanisms, which regulate the metabolic flux of substrates and intermediates in the tocochromanol pathway and provide a useful basis for future studies of the underlying regulatory mechanisms.

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