Engineering Plant Shikimate Pathway for Production of Tocotrienol and Improving Herbicide Resistance[1]

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Tocochromanols (tocopherols and tocotrienols), collectively known as vitamin E, are essential antioxidant components of both human and animal diets. Because of their potential health benefits, there is a considerable interest in plants with increased or customized vitamin E content. Here, we have explored a new strategy to reach this goal. In plants, phenylalanine is the precursor of a myriad of secondary compounds termed phenylpropanoids. In contrast, much less carbon is incorporated into tyrosine that provides *p*-hydroxyphenylpyruvate and homogentisate, the aromatic precursors of vitamin E. Therefore, we intended to increase the flux of these two compounds by deriving their synthesis directly at the level of prephenate. This was achieved by the expression of the yeast (*Saccharomyces cerevisiae*) prephenate dehydrogenase gene in tobacco (*Nicotiana tabacum*) plants that already overexpress the Arabidopsis *p*-hydroxyphenylpyruvate dioxygenase coding sequence. A massive accumulation of tocotrienols was observed in leaves. These molecules, which were undetectable in wild-type leaves, became the major forms of vitamin E in the leaves of the transgenic lines. An increased resistance of the transgenic plants toward the herbicidal *p*-hydroxyphenylpyruvate dioxygenase inhibitor diketonitril was also observed. This work demonstrates that the synthesis of *p*-hydroxyphenylpyruvate is a limiting step for the accumulation of vitamin E in plants.

Tocopherols and tocotrienols are amphiphilic lipids, collectively known as vitamin E. Albeit not abundant, they are essential components of both human and animal diets (Evans and Bishop, 1922; Epstein et al., 1966). Tocopherols are synthesized only by photosynthetic organisms (Lichtenthaler, 1968; Soll et al., 1985) through the condensation of an aromatic head group, which derives from homogentisic acid, and an isoprene chain, which arises from phytyldiphosphate. The four major forms of tocopherols differ in the position and number of methyl groups (Fig. 1). α-Tocopherol predominates in leaves of higher plants, whereas γ-tocopherol is often the major form in seeds (for review, see Munne-Bosch and Alegre, 2002). One of the best characterized functions of tocopherols is to protect biological membranes against oxidative stresses. The antioxidant properties of tocopherols arise from their ability to scavenge lipid peroxy radicals before they react with lipid substrates. These reactions produce tocopheroxyl radicals that are relatively unreactive and can be recycled by other antioxidants such as ascorbic acid and glutathione (Fryer, 1992). For human and animal health, α-tocopherol has the highest vitamin E activity. Vitamin E has long been implicated in preventing cardiovascular and neurodegenerative diseases and certain cancers. Furthermore, new functions as an antihypercholesterolemic and immunostimulatory agent have been proposed recently (for review, see Brigelius-Flohé et al., 2002). The amounts of vitamin E needed to achieve these effects are quite high (100–800 I.U.), whereas the recommended daily allowance is 40 I.U. Because of these health benefits, there is a considerable interest in plants with increased or customized tocopherol content. Attempts to reach this goal were achieved by overexpressing the structural genes involved in the biosynthesis of tocopherols. The first gene cloned in the tocopherol biosynthetic pathway, which codes for γ-tocopherol methyl transferase, was used to alter seed tocopherol composition (Shintani and DellaPenna, 1998). When this gene was overexpressed in Arabidopsis, γ-tocopherol, the predominant tocopherol of the seeds, was almost completely converted into α-tocopherol. However, the total tocopherol content remained unchanged (Shintani and DellaPenna, 1998). The homogentisate

[1] This work was supported by the Centre National de la Recherche Scientifique, by the Institut National de la Recherche Agronomique, and by Bayer CropScience, formerly Aventis CropScience.

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Article, publication date, and citation information can be found at http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.032441.

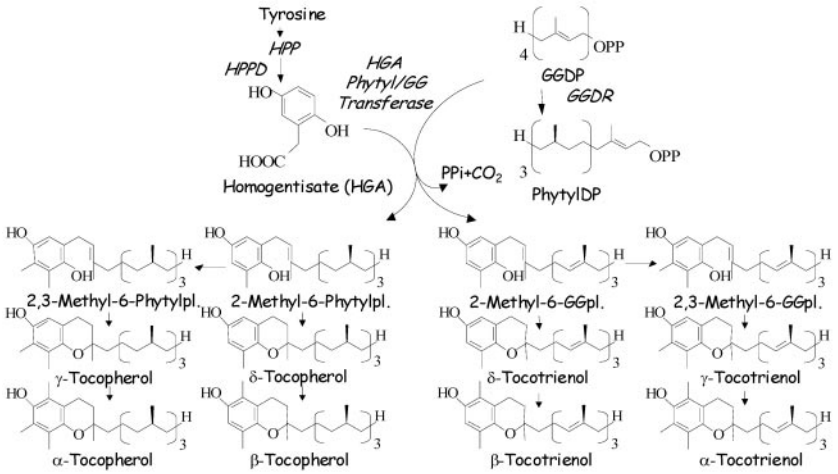

Figure 1. Tocopherol and tocotrienol biosynthetic pathway. PhytylDP, phytyldiphosphate; GGDP, geranylgeranyldiphosphate; GGDR, geranylgeranyldiphosphate reductase; 2,3-Methyl-6-Phytylpl., 2,3-methyl-6-phytylplastoquinol.

phytyltransferase gene encoding the first committed step in tocopherol biosynthesis was cloned recently from *Synechocystis* sp. PCC 68003 and Arabidopsis (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002). Its overexpression in Arabidopsis seeds increased tocopherol levels up to 80% (Savidge et al., 2002), and its overexpression in leaves increased tocopherol content up to 3- to 4-fold (Collakova and DellaPenna, 2003). Recently, overexpression of *p*-hydroxyphenylpyruvate dioxygenase (HPPD), the enzyme that catalyzes the transformation of *p*-hydroxyphenylpyruvate (HPP) into homogentisate, the aromatic precursor of tocopherols, was shown to have only a limited effect on tocopherol biosynthesis (Tsegaye et al., 2002; Falk et al., 2003).

In the present study, we explored a new strategy to increase plant vitamin E content based on the increase of HPP and homogentisate fluxes. In most plants, Phe and Tyr biosynthesis diverge at the level of arogenate, which is transformed into Tyr by arogenate dehydrogenase (EC 1.3.1.43) and into Phe by arogenate dehydratase (EC4.2.1.91; Fig. 2). Phe is the precursor of a myriad of secondary compounds termed phenylpropanoids. In certain circumstances, at least 30% of the carbon fixed during photosynthesis is incorporated into Phe for the synthesis of phenylpropanoids. The regulation of the shikimate pathway, in its post-chorismate section, reflects this situation. The last step in Tyr biosynthesis, catalyzed by arogenate dehydrogenase, is highly sensitive to the inhibition by Tyr, the product of the reaction (Rippert and Matringe, 2002). When it is required, this allows the cells to derive the majority of the carbon flux of the post-chorismate pathway for the synthesis of Phe and its phenylpropanoid derivatives. In contrast, much less carbon is incorporated into Tyr that provides HPP and homogentisate, the aromatic precursors of vitamin E. Thus, we intended to increase the HPP flux by deriving its synthesis directly at the level of prephenate, the precursor of arogenate (Fig. 2). This was achieved by the expression of a yeast (*Saccharomyces cerevisiae*) prephenate dehydrogenase (PDH), which catalyzes the transformation of prephenate into HPP, in tobacco (*Nicotiana tabacum*) plants that already overexpress an Arabidopsis HPPD (Garcia et al., 1999; Fig. 2).

The co-expression of yeast PDH in HPPD tobacco plants provoked a massive and quite unexpected accumulation of tocotrienols in leaves. These molecules, which were undetectable in wild-type leaves, become the major forms of vitamin E in the leaves of the transgenic lines. An increased resistance of the transgenic plants toward the herbicidal HPPD inhibitor diketonitril (DKN) also was observed.

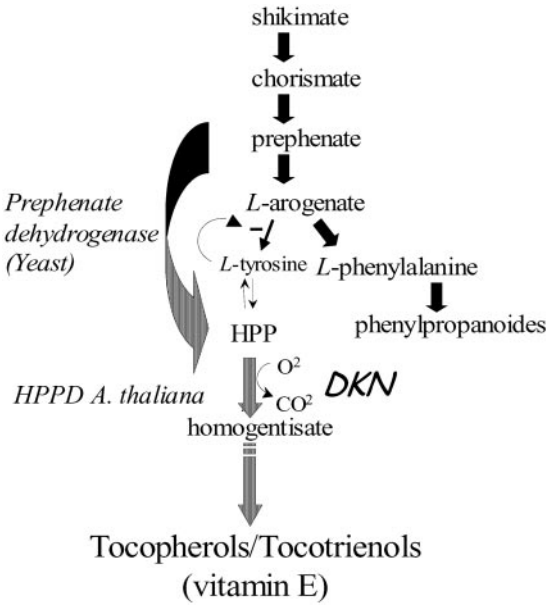

Figure 2. Phe, Tyr, and vitamin E biosynthesis from shikimate. The expected increase in HPP and homogentisate fluxes by the co-expression of yeast PDH and Arabidopsis HPPD are schematized by the large zebra arrows.

RESULTS

Cloning and Expression of the Recombinant Yeast PDH in *Escherichia coli* AT 2471 Cells

Yeast PDH catalyzes the oxidative decarboxylation of prephenate into HPP in the presence of NAD (Lingens et al., 1967). The complete coding region of the yeast structural gene for PDH *Tyr1*, previously described by Mannhaupt et al. (1989), was amplified by PCR from genomic DNA of the yeast W303α strain. The corresponding recombinant protein was overproduced in *E. coli* cells AT 2471 lacking endogenous PDH activity (Taylor and Trotter, 1967). Although recombinant yeast PDH could not be detected in the soluble crude extract of *E. coli* cells analyzed by SDS-PAGE stained with Coomassie Blue, a PDH activity of 500 to 700 units mg^{-1} was detected. The yeast enzyme activity was specific to prephenate and NAD (not shown).

Expression of the Yeast PDH in HPPD Tobacco Plants Led to Increased DKN Resistance

As mentioned previously, HPP flux available for tocopherol biosynthesis is quite limited in plants. To increase it, we decided to connect the synthesis of HPP directly to prephenate by overexpressing yeast PDH in HPPD tobacco plants (Fig. 2). Because in plants the aromatic amino acid biosynthetic pathway takes place in plastids (Bickel et al., 1978), the coding sequence of yeast PDH, a cytosolic enzyme (Mannhaupt et al., 1989), was fused to an optimized plastid transit peptide (OTP), deriving from the transit peptide of the small subunit of Rubisco (Lebrun et al., 1992).

Twenty-six different HPPD-PDH tobacco lines were generated by *Agrobacterium tumefaciens* transformation of the yeast PDH gene in tobacco plants, which already overexpress the Arabidopsis HPPD coding sequence. These transgenic HPPD tobacco plants were initially selected in the presence of 4 ppm of the HPPD inhibitor DKN, whereas wild-type tobacco plants are sensitive to 0.5 ppm DKN (Garcia et al., 1999). This was the first demonstration, to our knowledge, that overexpression of a plant HPPD confers resistance toward HPPD inhibitors. Therefore, because the yeast PDH construct confers kanamycin resistance, tobacco plants harboring the yeast PDH and Arabidopsis HPPD transgenes were selected on the basis of their ability to grow on medium containing 100 μg mL^{-1} kanamycin and 4 ppm of DKN. To determine whether the expression of yeast PDH increases the resistance to DKN, all the progenies were tested for their capacity to germinate and grow in the presence of increasing concentrations of DKN. All but one of the 26 HPPD-PDH tobacco lines (HPPD-PDH 4) were able to germinate, grow, and did not develop chlorotic symptoms in the presence of 10 ppm DKN. The three best resistant lines, HPPD-PDH 14, HPPD-PDH 18, and HPPD-PDH 24, could germinate and grow normally in the presence of DKN concentrations up to 32 ppm, i.e. 6- to 8-fold, which was tolerated by the transgenic HPPD tobacco plants, and more than 40- to 60-fold, which was tolerated by the wild-type plants (Fig. 3). The presence of PDH transcripts was then analyzed by quantitative PCR in these three best resistant lines and in the nonresistant HPPD-PDH 4 line. Quantitative PCR analysis of HPPD-PDH 14, HPPD-PDH 18, and HPPD-PDH 24 tobacco plants resistant to 32 ppm DKN revealed the presence of PDH transcripts. In contrast, the analysis of HPPD-PDH 4 tobacco plants could not reveal any expression of the PDH gene (not shown). This result explains why this line is unable to grow in the presence of DKN concentrations over 4 ppm, although it is resistant to kanamycin. Thus, these results confirm that the increase in DKN resistance is linked to the expression of yeast PDH. Analyses of three independent tobacco lines expressing only PDH revealed no increase in resistance to DKN versus wild-type tobacco (data not shown). This indicates that the overexpression of PDH alone is not sufficient to confer DKN resistance.

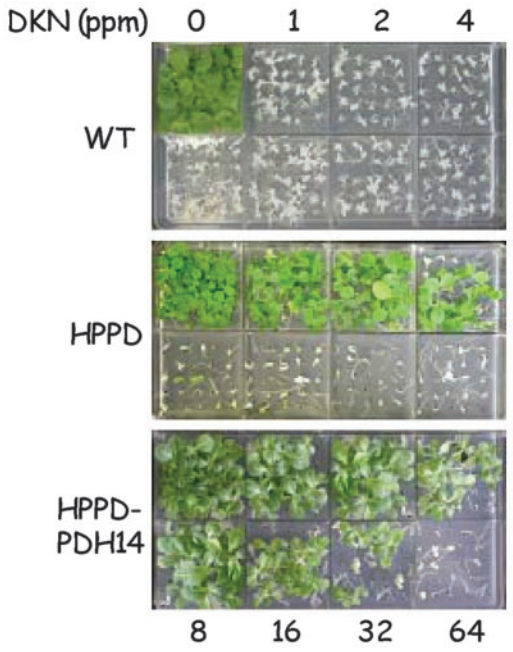

Figure 3. Resistance toward the HPPD inhibitor DKN. Wild type tobacco plants (WT), tobacco plants expressing the Arabidopsis HPDD, and tobacco plants co-expressing the Arabidopsis HPPD and the yeast PDH (HPPD-PDH 14) were allowed to grow on Murashige and Skoog medium in the presence of increasing concentrations of the HPPD inhibitor DKN (1–64 ppm). Wild-type tobacco plants could not grow in the presence of 1 ppm of DKN, and the transgenic HPPD tobacco plants could grow and did not develop chlorotic symptoms in the presence of DKN concentration up to 4 ppm. Some of the HPPD-PDH 14 T1 progeny could grow and did not develop chlorotic symptoms in the presence of DKN concentration up to 32 ppm.

Overexpression of Yeast PDH in HPPD Tobacco Plants Led to a Large Accumulation of Tocotrienol

The vitamin E content of the three best DKN-tolerant HPPD-PDH tobacco lines (HPPD-PDH 14, HPPD-PDH 18, and HPPD-PDH 24) was analyzed and compared with that of the wild-type tobacco plants PBD6, the transgenic HPPD tobacco plants, and the HPPD-PDH 4 tobacco line. Seeds from the HPPD-PDH 14, HPPD-PDH 18, and HPPD-PDH 24 tobacco lines were allowed to germinate in the presence of 32 ppm DKN and 100 μg mL^{-1} kanamycin. Seeds of the HPPD-PDH 4 tobacco line were germinated and grown in the presence of 100 μg mL^{-1} kanamycin and 4 ppm DKN. Two months after transfer to the greenhouse, young leaves were collected for vitamin E analyses. Tocopherol content was analyzed by HPLC on a C18 reverse phase column (see "Materials and Methods"). HPLC profiles and vitamin E contents from young leaves of the wild-type tobacco plants PBD6, the transgenic HPPD tobacco plants, and HPPD-PDH 4 were found to be very similar (Fig. 4; Table I). The major tocopherol detected in these plants was α-tocopherol. Traces amounts of β-/γ-tocopherols were also detected (Fig. 4B; Table I). In contrast, the HPLC profiles and vitamin E contents from young leaves of HPPD-PDH 14, HPPD-PDH 18, and HPPD-PDH 24 tobacco plants differed dramatically (Fig. 4C; Table I). They presented three additional major peaks with reduced retention times. These three additional peaks had the same retention times as authentic α-, β-/γ-, and δ-tocotrienol standards (Fig. 4, A and C), and they co-eluted perfectly with the tocotrienol standards in co-injection experiments (data not shown). These three additional peaks were collected, and their fluorescence characteristics were recorded. They had exactly the same emission and excitation fluorescence spectra as the tocotrienol standards, i.e. excitation and emission maxima at 292 and 325 nm, respectively. To definitely confirm that these additional peaks corresponded to tocotrienols, the HPLC fraction 3 from HPPD-PDH 18 tobacco leaves extract, which was the more abundant fraction (Fig. 4C; Table I), was collected, and it was mass determined by electrospray mass spectral analysis. As shown in Figure 5, in CH_3OH/NH_4OH: 100/0.1, the mass spectrum of this fraction gave a pseudomolecular ion peak at mass-to-charge ratio of 423.28 atomic mass unit $[M-H]^-$ (Fig. 5B), which is identical to the pseudomolecular ion peak of the standard α-tocotrienol (Fig. 5A). Higher amounts of β-/γ-tocopherols were also detected in the HPLC profile of these three best HPPD-PDH lines, but α-tocopherol content apparently was not affected (Fig. 4B; Table I). Furthermore, the accumulation of tocotrienol had no effect on the chlorophyll and carotenoid contents from young leaves of HPPD-PDH transgenic plants (Table II).

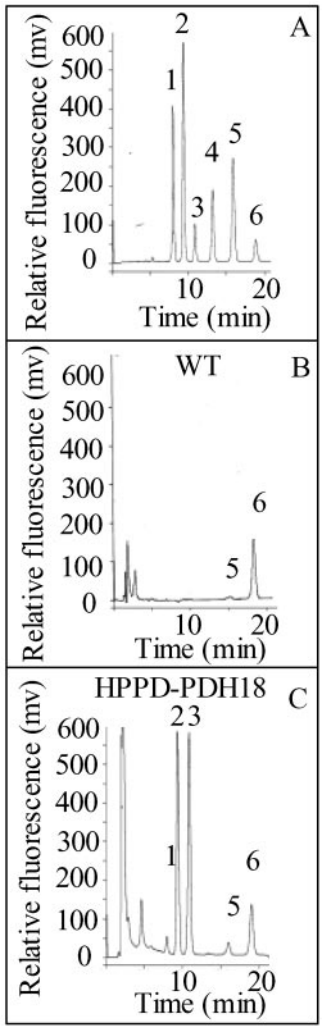

Figure 4. Representative HPLC analyses of vitamin E extracts from young leaves of wild-type and HPPD-PDH tobacco plants. A, HPLC profile of tocopherol and tocotrienol standards. Peak 1, δ-tocotrienol; peak 2, β-/γ-tocotrienol; peak 3, α-tocotrienol; peak 4, δ-tocopherol; peak 5, β-/γ-tocopherol; peak 6, α-tocopherol. B, Representative HPLC profile of a vitamin E extract from young leaves of wild-type tobacco plants (WT). Vitamin E extracts from young leaves of the transgenic HPPD tobacco lines and the HPPD-PDH 4 tobacco line gave similar HPLC profiles. C, HPLC profile of a vitamin E extract from young leaves of the HPPD-PDH 18 tobacco line. Vitamin E extracts from young leaves of the HPPD-PDH 14 and 24 tobacco lines gave similar HPLC profiles.

Tocotrienols could not be detected in the extracts of the wild-type tobacco and the transgenic HPPD tobacco plants or in those of the DKN-sensitive HPPD-PDH 4 line. Therefore, quantitative PCR analyses and measurement of vitamin E contents revealed that accumulation of tocotrienols occurred only in transgenic tobacco lines that expressed the yeast PDH gene. In HPPD-PDH tobacco plants, the three classes (α-, β-/γ-, and δ-tocotrienols) were detected, α-tocotrienol being the more abundant. Furthermore, their total tocotrienol content was nearly 10 times more abundant than their tocopherol content (497

Table I. *Tocopherols and tocotrienols contents of wild-type tobacco plants (WT), the transgenic HPPD tobacco plants (HPPD), the HPPD-PDH 4, HPPD-PDH 14, HPPD-PDH 18, and HPPD-PDH 24 tobacco lines, and the PDH 2 tobacco line*

Vitamin E	WT	HPPD	HPPD-PDH4	HPPD-PDH14	HPPD-PDH18	HPPD-PDH24	PDH2
	μg g^{-1} dry wt						
α-Tocopherol	53.5 ± 3.6	51.8 ± 3.6	54.7 ± 4.4	47.2 ± 3.2	48.3 ± 3.4	46.8 ± 3.1	51.2 ± 3.1
β/γ-Tocopherol	1.1 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	6.4 ± 0.7	6.5 ± 0.5	6.4 ± 0.6	1.4 ± 0.2
δ-Tocopherol	nd	nd	nd	nd	nd	nd	nd
α-Tocotrienol	nd	nd	nd	354.2 ± 13.6	412.3 ± 15.3	324.3 ± 11.7	14.3 ± 0.7
β/γ-Tocotrienol	nd	nd	nd	68.4 ± 4.2	78.3 ± 5.6	63.5 ± 4.4	nd
δ-Tocotrienol	nd	nd	nd	5.3 ± 0.3	6.2 ± 0.4	5.4 ± 0.4	nd
Total	54.6 ± 5	53.4 ± 3.7	52.3 ± 4.5	481.5 ± 22.1	551.6 ± 25.2	446.4 ± 20.2	67.5 ± 4

PDH 2 line contains only the yeast PDH transgene. nd, Not detected. Data are the mean of three independent analyses ± SD

and 55 μg g^{-1} dry material from young HPPD-PDH 18 leaves, respectively; Table I).

Analysis of our three independent tobacco lines expressing only the yeast PDH revealed that these lines exhibited little modification of their tocopherol HPLC profiles. However, small but nevertheless detectable amounts of α-tocotrienol were found in young leaves of these PDH tobacco plants (Table I; PDH 2). Although not necessary for tocotrienol synthesis in leaves, HPPD overexpression is required to obtain a large accumulation of tocotrienol because it is required for DKN resistance.

DISCUSSION

The aim of the present study was to deregulate the synthesis of both HPP and homogentisate by the co-expression of the yeast PDH and the Arabidopsis HPPD genes, expecting that this would improve the vitamin E content of the transgenic plants. Our results revealed that as expected, the co-expression of these two transgenes induced a dramatic modification of the vitamin E (tocopherols and tocotrienols) pattern of the transgenic plants and an improved resistance toward HPPD inhibitors. The expression of both transgenes is required to reach these effects. Tobacco plants expressing only the yeast PDH transgene accumulated only traces amounts of α-tocotrienol and presented the same sensitivity toward HPPD inhibitors as the wild-type plants, and the vitamin E pattern of HPPD tobacco plants was not modified.

The yeast PDH gene connects the synthesis of HPP directly at the level of prephenate. This resulted in an increase in HPP flux, which was evidenced in HPPD-

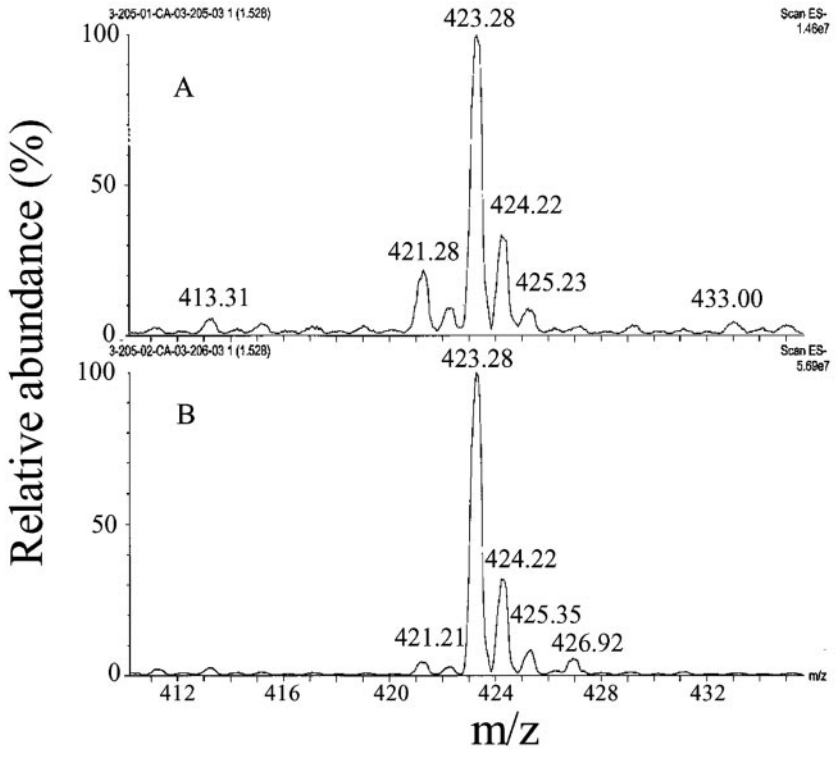

Figure 5. Mass spectrum analyses of α-tocotrienol. An α-tocotrienol standard (A) and the HPLC fraction 3 from a HPPD-PDH 18 tobacco vitamin E extract (B; see Fig. 4C) were dissolved in CH_3OH:NH_4OH (100:0.1 [w/v]) and introduced by electrospray into the Z source of a VG QUATTRO II instrument (Waters, Milford, MA). The tuning parameters were the following: source, capillary, 3.30 kV; cone, 42 V; source block temperature, 80°C; and desolvation temperature, 110°C. Both mass spectra give the same pseudomolecular ion peak at mass-to-charge ratio 423.28 atomic mass unit $[M-H]^-$, which is characteristic of α-tocotrienol.

Table II. *Chlorophyll and carotenoid contents from young leaves of wild-type and HPPD-PDH 14 tobacco plants*

mg per g of Dry Weight	WT	HPPD-PDH 14
Pigment	*mg g⁻ dry wt*	
Chlorophyll *a*	3.21 ± 0.46	3.56 ± 0.62
Chlorophyll *b*	0.87 ± 0.15	0.91 ± 0.17
Carotenoids	0.95 ± 0.15	0.99 ± 0.18

Data are the means of three independent analyses ± SD.

PDH transgenic tobacco plants by a higher resistance toward the HPPD inhibitor DKN. DKN is a competitive inhibitor of HPPD with respect to its substrate HPP (Ellis et al., 1996; Viviani Garcia et al., 1998; Garcia et al., 2000). Therefore, an increase of the concentration of HPP will prevent the binding of DKN at the active site of both native and recombinant HPPD. In accordance, HPPD-PDH tobacco plants had a 6- to 8-fold increase in DKN resistance with respect to the transgenic HPPD tobacco plants and a nearly 40- to 60-fold increase with respect to the wild-type plants.

More interestingly, the presence of the recombinant HPPD in the transgenic HPPD-PDH tobacco lines led to an increased homogentisate flux, which was used to produce a large amount of vitamin E. These transgenic lines had a vitamin E content 10- to 11-fold higher than the wild-type plants. Although only α-tocopherol and traces amounts of β-/γ-tocopherols could be detected in the wild-type plants, a large amount of tocotrienols was detected in the HPPD-PDH transgenic lines. These tocotrienols, which were undetectable in wild-type tobacco leaves, became the major forms in the transgenic plants, i.e. 10 times more abundant than tocopherols.

Tocotrienols differ from tocopherols by their isoprenoid side chain, which derives from geranylgeranyldiphosphate (geranylgeranylDP) instead of phytyldiphosphate (phytylDP) (Fig. 1). According to the literature, tocotrienols are found only in the seeds of some species, mostly monocotyledonous (for review, see Munne-Bosch and Alegre, 2002), and were never detected in leaves of all the plants analyzed so far except in *Cratoxylum sumatranum*, where some δ-tocotrienol was detected (Seo et al., 2002). Until now, the absence of tocotrienols in leaves suggested that any geranylgeranyl intermediate produced is either efficiently reduced in phytyl intermediate by geranylgeranyl-DP reductase, or that in leaves, geranylgeranyl-DP is not an available substrate for homogentisate phytyl transferase. In vitro studies have revealed that some of the enzyme activities involved in the biosynthesis of tocopherol, i.e. tocopherol methyl transferase and tocopherol cyclase, are active on geranylgeranyl intermediates (Soll and Schultz, 1979; Porfirova et al., 2002). In contrast, all the in vitro studies of plant homogentisate phytyl transferase activity failed to demonstrate any condensation of homogentisate with geranylgeranyl-DP (Hutson and Threlfall, 1980; Soll et al., 1980; Collakova and DellaPenna, 2001). During the reviewing process of our manuscript, the identification of a seed-specific homogentisate geranylgeranyl transferase from barley (*Hordeum vulgare*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) was reported (Cahoon et al., 2003). Overexpression of the barley enzyme in tobacco callus and Arabidopsis led to tocotrienol accumulation in transgenic material, whereas the non-transformed material never accumulated these compounds. Thus, this seed-specific enzyme explains the presence of tocotrienols in seeds and their absence in leaves of barley, rice, and wheat.

In the present study, the accumulation of tocotrienols in HPPD-PDH transgenic tobacco leaves is quite unexpected because it was obtained without any transgenic manipulation of a homogentisate geranylgeranyl transferase. Thus, our results demonstrate that in vivo, at least in tobacco, geranylgeranyl-DP could be a substrate for a chloroplastic homogentisate polyprenyl transferase and that all the subsequent enzymes of the pathway could accept geranylgeranyl intermediates because all four forms of tocotrienols (α, β/γ, and δ) were detected in young leaves of our transgenic HPPD-PDH tobacco lines. Tanaka et al. (1999) have reported that the down regulation of geranylgeranyl-DP reductase in tobacco did not result in tocotrienol accumulation, although it did shift the geranylgeranyl-DP to phytyl-DP ratio largely in favor of geranylgeranyl-DP. Furthermore, we did not detect important modification of the tocopherol content of our transgenic HPPD-PDH tobacco lines. Thus, the presence of tocotrienol in the leaves of these transgenic tobacco could not be explained by a depletion of their phytyl-DP pool. Rather, our results indicate that it was the increase in the HPP and homogentisate fluxes that provoked the accumulation of tocotrienols. We propose that in the transgenic HPPD-PDH tobacco lines, homogentisate concentration reached a level sufficient to allow the utilization of geranylgeranyl-DP by a chloroplastic homogentisate polyprenyl transferase in addition to its usual polyprenyl substrate. Alternatively, because tobacco seeds contain tocotrienols (Falk et al., 2003), one could not exclude that the pattern of expression of a putative endogenous seed-specific homogentisate geranylgeranyl transferase was modified in our transgenic PDH-HPPD tobacco, leading to the accumulation of tocotrienols in leaves. However, in a recent patent, Valentin and Mitzky (2002), using a strategy quite similar to ours, reported the accumulation of tocotrienols in Arabidopsis, a plant that usually never accumulated tocotrienols even in seeds and is apparently devoid of any seed-specific homogentisate geranylgeranyl transferase. Further studies are needed to confirm and explain the central role of homogentisate concentration in the regulatory mechanism preventing any accumulation of tocotrienols in leaves of nearly all plants.

Tocotrienols have a similar or even better antioxidant action than tocopherols (Kamal-Eldin and Appelqvist, 1996; Kamat et al., 1997). Furthermore, they present additional pharmaceutical interest in preventing cholesterol accumulation and, thus, arteriosclerosis by inhibiting 3-hydroxyl-3-methylglutaryl-CoA reductase (Qureshi et al., 1986; Pearce et al., 1992; Packer et al., 2001; Qureshi et al., 2001). It was also reported that tocotrienols have an antiproliferative action on cancer cells and act synergistically with tamoxifen against breast cancer (Guthrie et al., 1997). All these pharmaceutical properties of tocotrienols make tocotrienol-accumulating crops potentially very helpful for human health. Until now, all the attempts to manipulate the tocopherol pathway in Arabidopsis by overexpressing its structural genes (i.e. γ-tocopherol methyl transferase, homogentisate phytyltransferase, and HPPD; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002; Tsegaye et al., 2002) succeeded only in the alteration of the tocopherol content. Recently, Falk et al. (2003) reported a 2-fold increase in γ-tocopherol and γ-tocotrienol contents of tobacco seeds by overexpression of the HPPD from barley. However, in all these studies, tocotrienols were never detected in leaves of the corresponding transgenic lines. The report of Cahoon et al. (2003), the present work, and the recent patent (Valentin and Mitzky, 2002) are the first demonstrations, to our knowledge, that tocotrienols could accumulate in tissues that usually never accumulate these compounds. They demonstrate that different engineering strategies could be used to modulate and customize both the level and the pattern of tocotrienols in crop plants.

These tocotrienol-accumulating plants will also help us to analyze whether the presence of tocotrienols in their vegetative tissues confers new agronomical traits resulting from an expected higher tolerance toward oxidative stress. Several studies revealed a correlation between tocopherol content and resistance toward oxidative stresses from abiotic origin (high light, chilling, drought, salt stresses, and heavy metals; for review, see Munne-Bosch and Alegre, 2002). Finally, the increased tolerance toward HPPD inhibitor herbicides in addition to the accumulation of the high-value tocotrienol compounds in HPPD-PDH transgenic plants will make the weed control in future HPPD-PDH crop field much easier.

MATERIALS AND METHODS

Materials

Tocopherol and tocotrienol standards were obtained from Merck (Rahway, NJ). Isopropylthio-β-d-galactoside was purchased from Bioprobe Systems (Montreuil, France), and all other biochemicals were obtained from Sigma (St. Louis).

Bacterial Strains

The *tyrA Escherichia coli* AT 2471 strain (Taylor and Trotter, 1967) corresponding to the *thi1 tyrA* mutant was purchased from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). This mutant was lysogenized with the helper phage (λDE3) harboring a copy of the T7 RNA polymerase gene using the λDE3 lysogenization kit from Novagen (Madison, WI). The resulting *E. coli* AT 2471(DE3) could be used to express cDNAs cloned in the pET vectors under the control of the T7 promoter.

Plant Material

Tobacco (*Nicotiana tabacum*) var PBD6 plants were grown in soil under greenhouse conditions (23°C with a 16-h photoperiod and a light intensity of 200 μmol photons m^{-2} s^{-1}).

Cloning and Overexpression of Yeast (*Saccharomyces cerevisiae*) PDH in *E. coli*

Plasmid Engineering

The complete coding sequence of yeast PDH gene (*Tyr1*, accession no. Z36035) was amplified by PCR from genomic DNA of the yeast strain W 303 α with the following primers: P1, 5′-GATAACCATGGTATCAGAGGATAAGAT-3′ that introduces an *Nco*I restriction site (underlined); and P2, 5′-GAAGGCCTAATATTATAGGAAATCAGC-3′. The PCR product was cloned into pPCR Script (Stratagene, La Jolla, CA), yielding the plasmid pPCR Script-PDH, the *Nco*I-*Bam*H I fragment was subcloned into pET 23d (Novagen), yielding the plasmid pET 23-PDH. The cDNA insert was sequenced on both strands to ensure that no undesired mutations had been introduced during the course of the PCR amplification.

Overproduction of the Recombinant Yeast PDH

The *E. coli* AT 2471(λ DE3) cells transformed with pET23-PDH were grown at 37°C in Luria-Bertani medium supplemented with the appropriate antibiotics. When A_{600} reached 0.6, 1 mM isopropylthio-β-d-galactoside was added to induce the recombinant protein synthesis. The cells were further grown for 16 h at 28°C and harvested by centrifugation at 4,000*g* for 20 min. The pellet was resuspended in buffer A containing 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, and 500 mM NaCl, and sonicated with a Vibra-cell disrupter (100 pulses every 3 s on power setting 5, Sonics and Materials, Danbury, CT). The crude extract obtained was centrifuged for 20 min at 40,000*g*. Cell free supernatants were used for enzymatic assays.

Protein Concentration

Total protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) with γ-globulin as the standard, as described by Bradford (1976).

PDH Activity

PDH activity was assayed at 25°C according to Champney and Jensen (1970) by following the formation of NADH at 340 nm in 50 mM Tris-HCl (pH 7.5) in the presence of 1 mM prephenate and 1 mM NAD, in a total volume of 200 μL. Enzymatic activity is expressed as micromoles NADH formed per minute per milligram of protein (units per milligram).

Transformation of Tobacco Plants

Plasmid Engineering

The complete coding sequence of yeast PDH was amplified by PCR from the plasmid pPCR Script-PDH with the primers P2 (see above) and P3, 5′-*GCGGAA*GGATCCGGTGCATGGTATCAGAGGATAAGATTGAG-3′. Primer P3 is a fusion of the 17 last nucleotides of the optimized transit peptide (OTP; italic; Lebrun et al., 1992), in frame with the 24 first nucleotides of the coding sequence of the yeast PDH gene *Tyr1*. The OTP sequence

contains a *Bam*H I restriction site (underlined). The PCR product was cloned into pPCR Script (Stratagene), yielding the plasmid pPCR Script-OTP-PDH. A *Bam*H I-*Not*I DNA fragment was then subcloned into the plasmid pET21, yielding the plasmid pET21-OTP-PDH. A *Bam*H I-*Xho*I DNA fragment was then subcloned into the binary vector pRD 224, yielding the plasmid pRD 224-OTP-PDH. The OTP-PDH open reading frame is placed under the control of the duplicated promoter from the Arabidopsis histone gene H4748, the transcription activator from the tobacco etch virus, and the *Nos* terminator. Plasmid pRD 224 also carries the npt II gene that confers the kanamycin resistance to transformed plant cells.

Plant Transformation

Plasmid pRD 224-OTP-PDH was transferred by electroporation to *Agrobacterium tumefaciens* (strain EHA 105) and used for transformation of a tobacco cv PBD6-HPPD line by leaf disc transformation, according to Horsh et al., 1985. This tobacco line originally selected is a transgenic homozygous and monolocus line, which already overproduces the HPPD of Arabidopsis, the enzyme that catalyzes the transformation of HPP into homogentisate (Garcia et al., 1999). Transformed cells were selected on regenerating media containing 100 μg mL^{-1} kanamycin and 4 ppm of DKN, a potent HPPD inhibitor (Garcia et al., 1999). The presence of the PDH transgene was confirmed by PCR analysis.

Resistance of Transgenic Tobacco Plants toward HPPD Inhibitors

Transgenic plants co-expressing the Arabidopsis HPPD and the yeast PDH were selected by growing all the lines on Murashige and Skoog medium containing 100 μg mL^{-1} kanamycine and 4 ppm of DKN. DKN resistance of the selected transgenic tobacco lines was then evaluated by growing the T_1 progeny of transformed tobacco plants on Murashige and Skoog medium containing 100 μg mL^{-1} kanamycin and 0-to-64 ppm DKN. The resistance of tobacco plants overexpressing only one transgene, i.e. the Arabidopsis HPPD or the yeast PDH, was also tested as control.

Extraction of mRNA

Poly(A^+) mRNAs from young leaves of the different tobacco lines (T_1 generation) used for tocopherol analyses (see below) were prepared using Straight A's mRNA Isolation System according to the manufacturer's instructions (Novagen). First strand cDNA was synthesized from 1 μg of mRNA in a final volume of 20 μL using oligo$(dT)_{20}$ primers (Thermoscript RT-PCR System, Life Technologies/Gibco-BRL, Cleveland).

Real-Time PCR

The presence of yeast PDH transcripts in transgenic tobacco was determined by real-time reverse transcriptase-PCR using Lightcycler System (Roche Biomolecular Biochemicals, Basel, Switzerland) and Lightcycler-Faststart DNA Master SYBR Green I kit. For each measurement, 1 μL of cDNA prepared from young leaf poly(A^+) mRNA was used as a template in a standard 10-μL LightCycler PCR with appropriate primers (used at a final concentration of 1 μM) and 5 mM $MgCl_2$ concentration. Amplification and detection were performed using the following program: 95°C/8 min followed by 40 cycles of 95°C/10 s, 64°C/5 s, and 72°C/5 s. The translation initiation factor 4A gene (NeIF-4A10; Mandel et al., 1995) was used as constitutively expressed control gene and internal standard of mRNA integrity and cDNA preparation. The following primers were used for PCR amplification: IF4A10+, 5′ctgtgaggattcttgtaaagcg 3′; IF4A10−, 5′ggtccatgtctccgtgagttgc 3′; yPDH+, 5′ctcggttgtttcttttgcc 3′; and yPDH−, 5′ctagaatgactcagaatgg 3′. The specificity of the reaction was verified by melting curve analysis obtained by increasing temperature from 55°C to 95°C (0.1°C s^{-1}).

Vitamin E Analyses

For each transgenic line, the youngest leaves of 2-month-old tobacco plants (10 plants of T_1 generation) were harvested and freeze dried. Freeze-dried material (150 mg), ground in liquid nitrogen, was extracted three times with 2 mL of hexane in dim light and in the presence of argon to prevent the oxidation of vitamin E. The resulting supernatants were pooled, evaporated to dryness under argon, dissolved in 3 mL of methanol bubbled with argon, and stored at −80°C until analyzed. For tocopherol and tocotrienol determinations, 100 μL of each sample was injected through a C18 HPLC column (Spherisorb ODS2 5μ 4 × 250, Interchim, Montluçon, France). Vitamin E was detected by fluorescence using excitation at 290 nm and recording emission at 325 nm. Tocopherols and tocotrienols were quantified by comparison with standards (Merck). The HPLC system consisted of two 510 HPLC pumps and a 712 WISP autosampler (Waters), an HPLC UV detector, and an SFM 25 fluorescence detector (Kontron Instruments, Eching, Germany). The flow rate was 1 mL min^{-1} with CH_3OH:H_2O (96:4 [v/v]) as a solvent. Quantification of tocopherols and tocotrienols was carried out by measuring peak areas using chromatograph data system 450-MT2 software (Kontron). Peak areas were converted to micrograms of tocopherols or tocotrienols by comparison with standard curves.

Mass Spectrum Analysis

Mass spectrum analyses were performed using a VG QUATTRO II high-resolution instrument (Waters) equipped with a Z spray introduction source. Four milliliters of an HPPD-PDH 14 hexane extract equivalent to 300 mg of freeze-dried material was evaporated to dryness under argon and dissolved in 300 μL of methanol. This methanol extract was injected into a C18 HPLC column as described above. Fraction corresponding to peak 3 (see Fig. 4C) was collected, evaporated to dryness under argon, and further purified by rechromatography in the same conditions. The resulting fraction was evaporated to dryness resuspended with 300 μL of CH_3OH:NH_4OH (100:0.1 [w/v]) and introduced into a stainless capillary. The tuning parameters were as follows: source, capillary, 3.30 kV; cone, 42 V; source block temperature, 80°C; and desolvation temperature, 110°C. Mass spectra of standard α-tocotrienol (Merck) was also obtained under the same conditions.

Determination of Chlorophyll and Carotenoid Contents

The content of chlorophyll and carotenoid in wild-type plants and transgenic HPPD-PDH plants were determined in 80% (v/v) acetone as described by Lichtenthaler (1987). Fifty milligrams of the freeze-dried material, ground in liquid nitrogen, was extracted three time in 1 mL of 80% (v/v) acetone, and A470, A645, and A663 were recorded.

ACKNOWLEDGMENTS

We are grateful to Roland Douce, Claude Alban, Renaud Dumas, and Stéphane Ravanel for critical reading of the manuscript. We are very grateful to Bertrand Lefèvre for mass spectrum analyses, which were carried out in the Laboratoire de Biospectrometrie (Centre de Recherche du Service de Santé des Armées, La Tronche, France).

Received August 27, 2003; returned for revision September 16, 2003; accepted October 20, 2003.

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