Tocotrienols, the Unsaturated Forms of Vitamin E, Can Function as Antioxidants and Lipid Protectors in Tobacco Leaves

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Vitamin E is a generic term for a group of lipid-soluble antioxidant compounds, the tocopherols and tocotrienols. While tocotrienols are considered as important vitamin E components in humans, with functions in health and disease, the protective functions of tocotrienols have never been investigated in plants, contrary to tocopherols. We took advantage of the strong accumulation of tocotrienols in leaves of double transgenic tobacco (Nicotiana tabacum) plants that coexpressed the yeast (Saccharomyces cerevisiae) prephenate dehydrogenase gene (PDH) and the Arabidopsis (Arabidopsis thaliana) hydroxyphenylpyruvate dioxygenase gene (HPPD) to study the antioxidant function of those compounds in vivo. In young leaves of wild-type and transgenic tobacco plants, the majority of vitamin E was stored in thylakoid membranes, while plastoglobules contained mainly δ-tocopherol, a very minor component of vitamin E in tobacco. However, the vitamin E composition of plastoglobules was observed to change substantially during leaf aging, with α-tocopherol becoming the major form. Tocotrienol accumulation in young transgenic HPPD-PDH leaves occurred without any significant perturbation of photosynthetic electron transport. Tocotrienols noticeably reinforced the tolerance of HPPD-PDH leaves to high light stress at chilling temperature, with photosystem II photoinhibition and lipid peroxidation being maintained at low levels relative to wild-type leaves. Very young leaves of wild-type tobacco plants turned yellow during chilling stress, because of the strongly reduced levels of chlorophylls and carotenoids, and this phenomenon was attenuated in transgenic HPPD-PDH plants. While sugars accumulated similarly in leaves of wild-type tobacco plants turned yellow during chilling stress, because of the strongly reduced levels of chlorophylls and carotenoids, and this phenomenon was attenuated in transgenic HPPD-PDH plants. While sugars accumulated similarly in young wild-type and HPPD-PDH leaves exposed to chilling stress in high light, a substantial decrease in tocotrienols was observed in the transgenic leaves only, suggesting vitamin E consumption during oxygen radical scavenging. Our results demonstrate that tocotrienols can function in vivo as efficient antioxidants protecting membrane lipids from peroxidation.

The term “vitamin E” describes the biological activity of a group of structurally related compounds, the tocochromanols, in animals and humans. Vitamin E is considered to be an essential, lipid-soluble nutrient that functions as an antioxidant protecting polyunsaturated fatty acids against lipid peroxidation (Fukuzawa and Gebicky, 1983; Fryer, 1992; Brigelius-Flohe and Traber, 1999; Bramley et al., 2000). Natural vitamin E includes four tocopherols and four tocotrienols, which are synthesized exclusively by oxygenic photosynthetic organisms. Tocopherols consist of a chromanol ring and a 20-carbon tail derived from the aromatic compound homogentisate and phytyl diphosphate, respectively. Tocotrienols differ structurally from tocopherols by the presence of three trans-double bonds in the hydrocarbon tail, which derive from geranylgeranyl diphosphate.

The first step in vitamin E synthesis involves the production of homogentisate from hydroxyphenylpyruvate (HPP) in a complex enzymatic reaction involving HPP dioxygenase (HPPD; Fiedler et al., 1982; García et al., 1997; DellaPenna and Pogson, 2006). HPPD has a key location in the tocochromanol pathway and is an important activity regulating tocochromanol fluxes in plants. However, overexpression of HPPD in Arabidopsis (Arabidopsis thaliana) or tobacco (Nicotiana tabacum) brought about modest increases in seed and leaf tocopherols (Tesegaye et al., 2002; Falk et al., 2003). This is due to the fact that the flux to HPP is an additional limiting step that is tightly regulated by feedback inhibition of arogenate dehydrogenase by its product Tyr (Rippert and Matringe, 2002). Rippert et al. (2004) succeeded in bypassing this feedback inhibition by expressing in tobacco a yeast (Saccharomyces cerevisiae) prephenate dehydrogenase (PDH) that catalyzes HPP production directly from prephenate. Although PDH expression alone had minor effects on tocochromanol levels, the simultaneous expression...
of yeast PDH and Arabidopsis HPPD in tobacco resulted in a strong increase (up to 8-fold) in leaves, indicating that both steps together are limiting for vitamin E synthesis in tobacco. However, a surprising result was that the increased tocchromanols in PDH-HPPD overexpressors were almost entirely because of tocotrienols, which are normally produced in tobacco seeds but not in tobacco leaves. Indeed, in plants, tocopherols and tocotrienols are distributed differently between organs, with tocotrienols being synthesized in seeds of some species, mainly monocotyledonous (Horvath et al., 2006), by a seed-specific geranylgeranyl homogentisate transferase (Cahoon et al., 2003). According to Rippert et al. (2004), it is possible that the high homogentisate flux in the double transgenic plants somehow altered the substrate specificity of homogentisate phytol transferase so that it was able to use geranylgeranyl diphosphate as a cosubstrate, thus resulting in tocotrienol synthesis. Similarly, coexpression of a bacterial PDH and the Arabidopsis HPPD in soybean (Glycine max) was reported to cause a strong accumulation of tocotrienols in seeds (Karunananda et al., 2005). In Arabidopsis, expression of the barley (Hordeum vulgare) seed-specific homogentisate geranylgeranyl transferase under the control of the 35S promoter is another strategy that successfully led to tocotrienol accumulation in leaves (Cahoon et al., 2003).

α-Tocopherol is the most abundant form of vitamin E in nature, with the highest bioavailability in the human body (Traber and Sies, 1996). Vitamin E research, therefore, has mainly focused on this compound and has neglected the other vitamin E molecules. However, tocopherols and tocotrienols have similar antioxidant activities in vitro (Serbinova et al., 1991; Serbinova and Packer, 1994; Yoshida et al., 2003). Moreover, recent developments indicate that the members of the vitamin E family are not redundant with respect to their biological functions. For instance, in tobacco leaves, α-tocopherol and γ-tocopherol have been shown to provide protection against different stress conditions (Abbasi et al., 2007). In humans, tocotrienols have emerged as unsaturated vitamin E molecules with functions in health and disease that are clearly distinct from that of α-tocopherol (Theriault et al., 1999; Sen et al., 2006). While different functions of tocopherols have been identified and characterized in vascular plants and cyanobacteria (Trebst et al., 2002; Sattler et al., 2004; Havaux et al., 2005; Maeda et al., 2006; Munne-Bosch et al., 2007), the physiological role of tocotrienols is not yet documented in plants. Because of the considerable accumulation of tocotrienols in their vegetative organs, the HPPD-PDH double transgenic tobacco plants described above provide a unique material to investigate their antioxidative function in vivo. This study is centered on those tocopherol-biofortified plants, which are compared with the wild type under normal and photooxidative stress conditions. The results presented show that tocotrienols function in vivo as efficient protectors and lipid antioxidants.

RESULTS

Tocopherol and Tocotrienol Levels and Distribution

The major form of vitamin E in wild-type tobacco leaves was α-tocopherol (Fig. 1), but relatively small amounts of γ-tocopherol (Fig. 1) and traces of δ-tocopherol (data not shown) were also detected. As reported previously (Rippert et al., 2004), expression of yeast PDH in HPPD transgenic tobacco plants brought about a massive accumulation of tocotrienols in leaves (Fig. 1). Besides α-, γ-, and δ-tocopherol molecules, whose concentrations were close to the wild-type level, double transgenic leaves (lines 24-4 and 17-1) contained very high amounts of α- and γ-tocotrienols (Fig. 1) and traces of δ-tocotrienol (data not shown). A small increase in the γα-tocopherol ratio was observed, however, in HPPD-PDH transgenic leaves relative to wild-type leaves. The total vitamin E content (tocopherols + tocotrienols) of the double transgenic lines was increased by a factor of about 5 compared with the wild-type level, with tocotrienols representing about 85% of vitamin E. The tocotrienol level reached by transgenic leaves (approximately 0.6 mg g⁻¹ dry weight) was comparable to that reported by Cahoon et al. (2003) in Arabidopsis plants overexpressing barley homogentisate geranylgeranyl transferase.

Intact chloroplasts isolated from young developing tobacco leaves by centrifugation on a Percoll gradient were fractionated into three fractions by ultracentrifugation on a Suc gradient (Supplemental Fig. S1A), which correspond to the three lipid structures of the chloroplast: the thylakoid membranes, the envelope membranes, and the plastoglobules (Lichtenthaler, 1968). The green pellet at the bottom of the tube contained the thylakoid membranes, while the yellowish band in the upper part of the tube contained plastoglobules. The third band between the plastoglobules and the thylakoids was strongly enriched in envelope membranes. The purity of the preparations was controlled by Western blots of proteins specific to each fraction.

Figure 1. Vitamin E content (tocopherols and tocotrienols) of wild-type and double transgenic HPPD-PDH tobacco leaves (lines 17-1 and 24-4) grown under control conditions (70 μmol m⁻² s⁻¹ and 25°C). D.W., Dry weight. Data are mean values of three separate experiments ± se.
The major chlorophyll-binding antenna protein light-harvesting complex protein (LHCP) was found exclusively in the thylakoid fraction, as expected. ceQORH, a protein of the inner envelope membrane (Miras et al., 2002), was present in the envelope-enriched fraction only. None of those proteins was found in the plastoglobules, confirming the purity of this fraction. Tocopherol cyclase VTE1, a plastoglobule marker, was detected in the plastoglobules and also in the envelope-enriched fraction. Since the chloroplast envelope is supposed to be derived from tocopherol cyclase activity (Soll et al., 1985; Vidi et al., 2006), we speculate that the envelope fraction was contaminated by plastoglobules. Accordingly, when this fraction was washed and recentrifuged, VTE1 was not detected anymore in the resulting purified fraction, while ceQORH was still present (Supplemental Fig. S1C).

Total lipids of each chloroplast fraction were extracted with a chloroform-methanol mixture according to Folch et al. (1957), and the vitamin E content of each fraction was determined by HPLC (Fig. 2) using standards of the eight vitamin E compounds (α-, β-, γ-, and δ-tocopherols and -tocotrienols; Supplemental Fig. S2). Figure 2 (right) summarizes the quantification of the vitamin E distribution in each lipid fraction. Thylakoids of wild-type chloroplasts were enriched in α-tocopherol and contained also γ-tocopherol, with the α:γ-tocopherol ratio (approximately 15:1) being close to that measured in leaves or in intact chloroplasts (Fig. 2). δ-Tocopherol represented less than 1% of vitamin E in this fraction. In contrast, the plastoglobule fraction contained mainly δ-tocopherol (approximately 90%). The vitamin E content of the envelope-enriched lipid fraction appeared to be intermediate between that of plastoglobules and thylakoids, with a mixture of δ-tocopherol and α-tocopherol. The same pattern was observed in transgenic chloroplasts: the major form of vitamin E in plastoglobules was δ-tocopherol, whereas the vitamin E profile of the thylakoid membranes (i.e. α-tocopherol and the different forms of tocotrienols) was similar to that measured in leaves and intact chloroplasts (Fig. 3).

**Photosynthetic Electron Transport**

The quantum yield of PSII-mediated electron transport was measured by chlorophyll fluorometry at different photon flux densities (PFDs), and no significant difference was found between wild-type and transgenic plants (Fig. 4A). Neither the maximal photochemical efficiency of PSII (Fv/Fm; measured at PFD = 0 in Fig. 4A) nor the light saturation curve of the electron transport quantum yield were changed in tocotrienol-accumulating plants relative to wild-type plants. We also measured photosynthetic oxygen evolution at different PFDs using a Clark electrode (Fig. 4B). Again, transgenic leaves could not be distinguished from wild-type leaves. Therefore, we can conclude that massive accumulation of tocotrienols in thylakoid membranes of young HPPD-PDH leaves did not perturb the photochemical activity.

**Levels of Antioxidants in HPPD-PDH Transgenic Tobacco Leaves**

Engineering of soybean using Arabidopsis HPPD and bacterial PDH has been reported to cause large...
increases in tocotrienols in seeds but also massive accumulation of the precursor homogentisate (Karunananda et al., 2005). This was not the case in our transgenic plants: the homogentisate level, measured using HPLC (Garcia et al., 1997), was below the detection limit (\( \leq 1 \) nmol) in both wild-type and HPPD-PDH leaves (data not shown). Similarly, the carotenoid level was not modified in the HPPD-PDH leaves (Rippert et al., 2004).

We also measured two major antioxidant compounds, ascorbate and glutathione (Fig. 5, A and B), and the abundance of a number of antioxidant enzymes, peroxiredoxin Q (Prx Q), 2-Cys peroxiredoxin (2-Cys Prx), and Met sulfoxide reductase A4 (MsrA4; Fig. 5C). Prxs can detoxify organic peroxides (Dietz, 2003), while MsrS regenerate Met sulfoxide back to Met and are thus involved in protein repair during oxidative stress conditions (Rouhier et al., 2006). None of those antioxidant systems was significantly affected by tocotrienol accumulation. Moreover, the reduction levels of both ascorbate and glutathione were similar (approximately 90%) in wild-type and transgenic plants. Similarly, the ratio between reduced and oxidized MsrA4 (Veira Dos Santos et al., 2005) and the amount of overoxidized Cys in 2-Cys Prx (i.e. the level of Cys sulfenic acid form; compare with Rey et al. [2007]) were unchanged in the transgenic plants. Thus, tocotrienol accumulation did not seem to significantly perturb the antioxidant machinery of the chloroplasts.

**Tolerance to Photooxidative Stress and PSII Photoinhibition**

Tobacco plants grown under control conditions (\( 25 \degree C, 150 \mu mol \) photons \( m^{-2} s^{-1} \)) were suddenly exposed to a higher PFD (700 \( \mu mol \) photons \( m^{-2} s^{-1} \)) at low temperature (\( 10 \degree C \)). The combination of light and low temperature is very favorable for inducing photooxidative stress, especially in chilling-sensitive plants such as tobacco (Wise, 1995). After 4 d of such treatment, lipid peroxidation and oxidative stress were visualized by imaging leaf autoluminescence. The latter technique measures the faint light emitted by singlet oxygen and triplet carbonyls, the by-products of the slow spontaneous decomposition of lipid hydroperoxides and endoperoxides (Devaraj et al., 1997; Vavilin and Ducruet, 1998; Havaux et al., 2006). Deactivation of excited carbonyls and singlet oxygen produces photons in the blue and red spectral regions, respectively. The latter photon emissions, although very weak, can be recorded with a high-sensitivity, liquid nitrogen-cooled CCD camera (Havaux et al., 2006). This technique has been used to map lipid peroxidation and oxidative stress in various biological materials, including detached leaves (Flor-Henry et al., 2004), whole plants (Johnson et al., 2007; Collin et al., 2008), animals (Kobayashi et al., 1999), and humans (van Wijk et al., 2006). As shown in plants aged 25 or 40 d (Fig. 6, A and B, respectively), spontaneous photon emission by wild-type plants was noticeably enhanced relative to HPPD-PDH plants after high light stress at low temperature. Photon emission was greatest in the oldest wild-type leaves. In transgenic leaves, spontaneous photon emission was weak, being essentially restricted to the margins of some leaves.

Thermoluminescence (TL) provides another convenient method for quantifying lipid peroxidation (Havaux, 2003). This method consists of slowly heating the leaf sample to \( 150 \degree C \) to provoke the thermal
Lipid peroxidation was also analyzed by quantifying lipid hydroperoxides using HPLC. As shown in Figure 8A, the concentration of lipid hydroperoxides produced by the action of reactive oxygen species (ROS) increased in wild-type leaves after high light stress. This increase was not found in transgenic leaves, confirming their resistance to photooxidative stress. We also determined the level of lipid hydroperoxides catalyzed by lipoxygenases. We did not find that light stress caused the accumulation of lipoxygenase-dependent peroxides in wild-type or transgenic plants (Fig. 8B), indicating that lipoxygenase is not involved in the response of tobacco to high light at low temperature.

ROS-induced lipid peroxidation in tobacco plants was accompanied by a partial inactivation of the photosystems embedded in the thylakoid membranes. In the wild type, $F_v/F_m$ dropped from approximately 0.8 to 0.32 after 3 d and to 0.22 after 6 d in high light at cold temperature (Fig. 9A). PSII was less affected in the transgenic plants, with $F_v/F_m$ stabilizing at around 0.55 to 0.6. Moreover, PSII recovered fully (with $F_v/F_m$ of approximately 0.8) in the HPPD-PDH transgenic leaves within 6 to 7 h after removal of the stress conditions (Fig. 9B). In wild-type leaves, PSII recovery was slow, and the inhibition was reversed only partially after 7 h of reacclimation to low light at 25°C ($F_v/F_m$ of approximately 0.6); recovery was still incomplete ($F_v/F_m = 0.73 ± 0.03$) after 24 h in low light.

Acclimation of Young Leaves to High Light Stress at Low Temperature

Young tobacco leaves have been shown to be more resistant to photooxidation than mature, well-developed leaves (Havaux et al., 2003). Under the stress conditions used in this study, young leaves did not exhibit symptoms of oxidative stress (Fig. 10A), even after long-term exposure (>15 d), and this is confirmed by the autoluminescence images shown in Figure 6. The intensity of spontaneous photon emission was most intense in the most developed leaves: autoluminescence was high in wild-type plants aged 40 d compared with wild-type plants aged 25 d, and in the latter plants the very young leaves at the center of the plants had a very low luminescence, hardly measurable by our imaging technique. These observations were corroborated by TL measurements that failed to show increases in HTL band amplitude in young leaves after light stress (data not shown). Although young leaves were photoresistant and were able to grow under the stress conditions, they were very pale (Fig. 10A), particularly in wild-type plants, due to a marked decrease in the chlorophyll concentration (Fig. 10B). Interestingly, young leaves of transgenic plants were much greener than wild-type leaves (Fig. 10A), and, as expected, this was associated with a higher chlorophyll content (Fig. 10B). The carotenoids followed the same trend: their concentration was reduced with light stress to a greater extent in young leaves.
wild-type leaves relative to HPPD-PDH leaves (Fig. 11A), except for zeaxanthin, which accumulated in the wild type (Fig. 11B). Zeaxanthin synthesis is induced by conditions of excessive light energy (Demmig-Adams and Adams, 2000), suggesting that wild-type leaves sensed a higher level of light stress than HPPD-PDH leaves. We also determined the sugar level (starch and soluble sugars) after long-term stress, since vitamin E is known to be involved in photoassimilate transport (Russin et al., 1996; Hofius et al., 2004; Maeda et al., 2006). Stress conditions brought about a significant increase in the sugar concentration, and the effect was very pronounced for starch (Table I). However, we did not observe any difference between wild-type and transgenic plants. The vitamin E level of young wild-type leaves remained stable during acclimation to high light and low temperature (Fig. 11C). In contrast, a substantial decrease (approximately 30%) in vitamin E was observed in stressed HPPD-PDH leaves, mainly because of tocotrienol decrease (Fig. 11D). Nevertheless, the total vitamin E level remained higher in young transgenic leaves relative to wild-type leaves.

DISCUSSION

Tocotrienols Accumulated in the Thylakoid Membranes of Transgenic Tobacco Leaves and Did Not Perturb the Photochemical Activity of Chloroplasts

Tocotrienols are much less widespread in the plant kingdom than tocopherols (Horvath et al., 2006). In contrast to tocopherols, which are synthesized and accumulate in leaf plastids, tocotrienols are present in nongreen tissues, including seeds, fruits, and latex. However, plants can be genetically manipulated to synthesize and accumulate tocotrienols in leaves (Cahoon et al., 2003; Rippert et al., 2004). As shown here, high levels of tocotrienols can accumulate in modified tobacco leaves overexpressing yeast PDH and Arabidopsis HPPD, and this occurred without any significant perturbation of the leaf photochemical activity. Furthermore, tocotrienols had no effect on the chlorophyll and carotenoid contents of leaves (Rippert et al., 2004).

Inside chloroplasts, vitamin E is distributed between thylakoid membranes, envelope membranes, and plastoglobules (Lichtenthaler, 1969, 2007; Lichtenthaler et al., 1981; Vidi et al., 2006). However, only tocotrienols have been found to be involved in photoassimilate transport (Russin et al., 1996; Hofius et al., 2004; Maeda et al., 2006). Stress conditions brought about a significant increase in the sugar concentration, and the effect was very pronounced for starch (Table I). However, we did not observe any difference between wild-type and transgenic plants. The vitamin E level of young wild-type leaves remained stable during acclimation to high light and low temperature (Fig. 11C). In contrast, a substantial decrease (approximately 30%) in vitamin E was observed in stressed HPPD-PDH leaves, mainly because of tocotrienol decrease (Fig. 11D). Nevertheless, the total vitamin E level remained higher in young transgenic leaves relative to wild-type leaves.
in chloroplasts prepared from young wild-type leaves, in which the major forms of vitamin E were α- and γ-tocopherols in thylakoid membranes (and in leaves), while the plastoglobule fraction contained mainly δ-tocopherol. Thus, in young tobacco leaves, in which plastoglobules are present in small amounts, vitamin E was not stored predominantly in plastoglobules. Although α-tocopherol (and tocotrienols in the transgenic lines) was found in the envelope-enriched fraction, envelope membranes are assumed to represent around 5% to 6% of chloroplast membrane lipids (M. Block, personal communication) and therefore constitute a minor site of vitamin E storage relative to thylakoid membranes. For each fraction, vitamin E was normalized to the lipid content (data not shown), so that the distribution of vitamin E within the chloroplast could be estimated: we found 89%, 8%, and 3% for the thylakoids, the envelopes, and the plastoglobules, respectively.

This thylakoid localization fits with the function of vitamin E in plant leaves, which protects, in collaboration with the xanthophyll cycle (Havaux et al., 2005) and other antioxidant systems (Kanwischer et al., 2005), the thylakoid membrane lipid phase from phototoxidative damage. This finding is in apparent contradiction to a recent work by Vidi et al. (2006), who identified the plastoglobules as a major site of vitamin E accumulation in Arabidopsis chloroplasts. It is unlikely that the difference was due to the purification procedure, since the protocols used in both studies...
were very similar, except that we did not perform ultracentrifugation prior to the Suc gradient. This is to reduce plastoglobule losses and to improve vitamin E quantification in this fraction, since chloroplasts of our plants contain very low amounts of plastoglobules. In order to exclude the possibility that the contradictory results obtained in tobacco in this study and in Arabidopsis by Vidi et al. (2006) are due to the species used as starting material, we prepared plastoglobules from young Arabidopsis leaves. Again, we found δ-tocopherol as the major tocopherol and very little α-tocopherol in this fraction (data not shown). Plastoglobules are known to fluctuate during chloroplast development. They enlarge during thylakoid disassembly in senescing chloroplasts (Lichtenthaler, 1968; Tuquet and Newman, 1980; Ghosh et al., 2001) and under stress conditions (Locy et al., 1996; Eymery and Rey, 1999; Bondada and Syvertsen, 2003; Lichtenthaler, 2007). It is thus likely that the discrepancy in terms of plastoglobule composition between the two studies resulted from differences in the age and growth conditions of the plants used as experimental material.

Accordingly, when old and senescing leaves were used in our preparations, plastoglobules were more abundant and their vitamin E content was modified relative to that of young leaves, with α-tocopherol being the major vitamin E form (71%; Supplemental Fig. S3), as found by Vidi et al. (2006). However, they still contained δ-tocopherol (23%).

In good agreement with Vidi et al. (2006) and Austin et al. (2006), VTE1 was immunodetected in the plastoglobules of tobacco, confirming the occurrence of an active vitamin E synthesis at this level. Plastoglobules thus appear to have a dual function with respect to vitamin E: they actively participate to vitamin E synthesis via the tocopherol cyclase VTE1 and, under

Figure 8. HPLC measurements of lipid peroxidation in leaves of wild-type and transgenic plants exposed for 4 d to high light at low temperature (10°C, 700 μmol m⁻² s⁻¹, 14-h photoperiod). A, Concentrations of lipid hydroperoxides formed by ROS. B, Concentrations of lipid hydroperoxides generated enzymatically by lipoxygenases (LOX). F.W., Fresh weight. Data are mean values of three separate experiments ± sd.

Figure 9. A, Fv/Fm in wild-type and transgenic tobacco leaves before and after 3 or 6 d in high light at low temperature. Data are mean values of five separate experiments ± sd. B, Recovery of PSII photochemical activity after high light stress at low temperature. After 3 d in high light at low temperature, plants were transferred to low light at 25°C. Data are mean values of four or five measurements ± sd.
certain conditions, they also participate in vitamin E storage. In the study by Vidi et al. (2006), it seems likely that the second role of plastoglobules (i.e. vitamin E storage) was predominant. In contrast, the storage function of plastoglobules was very limited in the young tobacco leaves examined here. The partition of vitamin E biosynthesis between the chloroplast envelope and plastoglobules, and the accumulation of vitamin E in thylakoid membranes and, under certain conditions, in plastoglobules, highlight the very complex trafficking of tocopherols inside the chloroplast. However, it is clear from our data that there are conditions in which vitamin E is located predominantly in the thylakoid membranes.

The presence of δ-tocopherol as a major vitamin E component of plastoglobules is intriguing. This observation is in agreement with the localization of VTE1 in plastoglobules (Austin et al., 2006; Vidi et al., 2006; this study), since δ-tocopherol is one of two possible products of the cyclase reaction (Porfirova et al., 2002; Sattler et al., 2003). However, this finding also suggests that, in vivo, 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ), rather than 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ), could be the substrate of the tobacco tocopherol cyclase. As a corollary, the substrate of VTE3 (MPBQ methyltransferase) should be δ-tocopherol instead of MPBQ, leading to the formation of γ-tocopherol. However, these conclusions are difficult to reconcile with the biochemical characterization of Arabidopsis VTE1 and VTE3. In vitro, VTE1 presents a strong selectivity for DMPBQ (Porfirova et al., 2002; Sattler et al., 2003), and VTE3 was unable to methylate δ-tocopherol (Cheng et al., 2003). Moreover, DMPBQ, and not MPBQ, accumulated in the Arabidopsis vte1 mutant deficient in tocopherol cyclase (Maeda et al., 2006). Similarly, the massive accumulations of tocotrienol in transgenic HPPD-PDH tobacco leaves (Rippert et al., 2004) and Arabidopsis seeds (Karunanandaa et al., 2005) were also difficult to reconcile with the biochemical characterization of homogentisate phytyltransferase (HPT; Collakova and DellaPenna, 2001). One could argue that VTE1, VTE3, and HPT are all integral membrane proteins operating in a lipid environment and that such conditions are probably difficult to reproduce in vitro. Moreover, analysis of the tocopherol composition of Arabidopsis mutants deficient in VTE3 activity confirmed that, in planta, Arabidopsis VTE1 can utilize MPBQ, since δ- and β-tocopherols were found to accumulate in these mutants (Cheng et al., 2003). As far as the Arabidopsis vte1 mutant is concerned, it is possible that the pressure of the metabolic flux when the tocopherol synthesis is completely blocked by the lack of VTE1 can “force” the synthesis of DMPBQ from MPBQ by a methyltransferase, causing accumulation of the latter compound. The presence of δ-tocopherol in tobacco (and Arabidopsis) plastoglobules is an intriguing observation that cannot be fully understood in the light of the available literature, hence raising questions about vitamin E synthesis in plants.
aspect is beyond the scope of this article and will be studied further in the future.

Tocotrienols Function in Vivo as Efficient Antioxidants and Photoprotectors in Thylakoid Membranes

In vitro, tocotrienols have been shown to be better antioxidants than α-tocopherol (Serbinova et al., 1991; Suzuki et al., 1993). In animal and human cells, tocotrienols were reported to work in vivo as antioxidants at least as efficiently as tocopherols (Sen et al., 2006). Besides, tocotrienols possess powerful neuroprotective, anticancer, and cholesterol-lowering properties, which do not seem to be shared with α-tocopherol (Theriault et al., 1999; Sen et al., 2006). Thus, tocotrienols are considered to be major protective molecules in animal and human cells, with important bioactivities. In contrast, the antioxidant function of tocotrienols is not documented in plants. This is probably due to the fact that tocotrienol distribution is restricted essentially to seeds. Possibly, this peculiar distribution of tocotrienols may have hampered the study of their antioxidative activity, contrary to tocopherols, which are present in large quantities in leaves. The role of tocopherols in the protection of leaves against high light stress at low temperature (Havaux et al., 2005)

Table 1. Concentration of soluble sugars and starch (in mg g⁻¹ fresh weight) in young leaves of wild-type and HPPD-PDH transgenic plants exposed for 15 d to high light stress at low temperature (10°C and 700 μmol m⁻² s⁻¹)

<table>
<thead>
<tr>
<th>Material</th>
<th>Wild Type</th>
<th>HPPD-PDH 17-1</th>
<th>HPPD-PDH 24-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Stress</td>
<td>Control</td>
</tr>
<tr>
<td>Glc</td>
<td>1.43 ± 0.17</td>
<td>2.25 ± 0.17</td>
<td>3.12 ± 0.53</td>
</tr>
<tr>
<td>Fru</td>
<td>0.56 ± 0.07</td>
<td>0.78 ± 0.16</td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td>Saccharose</td>
<td>2.82 ± 0.37</td>
<td>5.61 ± 0.60</td>
<td>5.27 ± 0.45</td>
</tr>
<tr>
<td>Starch</td>
<td>2.13 ± 0.80</td>
<td>27.9 ± 1.05</td>
<td>2.43 ± 0.67</td>
</tr>
</tbody>
</table>

Leaf water content was not different in wild-type and HPPD-PDH plants. Data are mean values of three experiments ± SD.

Figure 11. Concentration of total carotenoids (A), zeaxanthin (B), tocopherols (C), and tocotrienols (D) in young wild-type and transgenic tobacco leaves before (white bars) and after long-term exposure (15 d) to high light at low temperature (black bars). Data are mean values of three to five experiments ± SD. Tocopherol and tocotrienol levels are semiquantitative and not adjusted for losses during extraction.
and salt or sorbitol stress (Abbasi et al., 2007) was recently demonstrated in tocopherol-deficient mutants. Similarly, tocopherols or their immediate precursors were observed to limit lipid peroxidation during germination in the light and during the early photoautotrophic growth of young seedlings (Sattler et al., 2004).

Because they accumulate tocotrienols in their leaves, our HPPD-PDH transgenic plants provided a unique opportunity to study in vivo the function of tocotrienols (e.g. under light-induced oxidative stress). When wild-type tobacco plants were exposed to high light and low temperature, they suffered from photooxidative stress and extensive lipid peroxidation, as measured by a variety of methods (HPLC, TL, and autoluminescence imaging). Compared with wild-type leaves, tocotrienol-accumulating plants were more phototolerant, showing no or very little lipid peroxidation and PSII photodamage. The differential phototolerance of wild-type and HPPD-PDH transgenic leaves provides clear evidence for the antioxidative potency of tocotrienols against peroxidation of membrane lipids. It is very unlikely that the increased phototolerance of the HPPD-PDH leaves was indirectly due to secondary changes in other antioxidant systems. No significant change in antioxidant molecules, such as ascorbate, glutathione, and carotenoids, or in antioxidant enzymes, such as Prx and Msr, was detected in the tocotrienol-accumulating transgenic leaves. We can also exclude the notion that the small changes in the α-tocopherol content and in the γα-toctopherol ratio were involved in the increased resistance of the HPPD-PDH plants to oxidative stress. Indeed, the Arabidopsis vte1 mutant, which lacks tocopherols (Porfirova et al., 2002), and the vte4 mutant, in which α-tocopherol is almost completely substituted by γ-tocopherol, did not exhibit a significant increase (or decrease) in phototolerance (Porfirova et al., 2002; Bergmüller et al., 2003; Havaux et al., 2005).

Moreover, tocotrienol accumulation enhanced the ability of young transgenic leaves to acclimate to high light stress in the long term. It has been shown in various plant species, including tobacco, that young, developing leaves are more tolerant to photooxidative stress than mature, well-developed leaves (Carlsson et al., 1996; Havaux et al., 2003). Although young tobacco leaves were able to tolerate the stress conditions used in this study, their pigmentation was strongly perturbed, leading to pale green/yellow leaves. This finding is consistent with previous studies on other thermophilic species, which have shown that chloroplast biogenesis is impaired by chilling stress (Nie and Baker, 1991; Yoshida et al., 1996). This phenomenon was attenuated in HPPD-PDH double transgenic leaves, which were greener and contained more chlorophyll than young wild-type leaves. Thus, chloroplast biogenesis was less perturbed by chilling stress when tocotrienols were able to accumulate in the chloroplasts.

The origin of leaf chlorosis under chilling stress is elusive. One possible cause is the accumulation of photoassimilates (Strand et al., 1997), a phenomenon that is known to repress photosynthetic gene expression (Sheen, 1990) and to cause the down-regulation of chlorophyll level (Braun et al., 2006). Importantly, tocopherol deficiency has been reported to lead to cold-induced blockage of photoassimilate transport in Arabidopsis (Maeda et al., 2006). Although sugars and starch did accumulate in young tobacco leaves exposed to chilling stress in high light, these accumulations were similar in wild-type and HPPD-PDH transgenic leaves.

Leaf chlorosis could also result from chilling-induced changes in the physical properties of the thylakoid membranes. A primary effect of chilling stress in thermophilic species is believed to be a decrease in membrane fluidity associated with the transition of membrane lipids from a flexible liquid crystalline to a solid gel phase (Murata et al., 1975; Wada et al., 1990; Nishida and Murata, 1996). In tobacco, phase separation was measured to occur below around 10°C (Terzaghi et al., 1989). Chloroplast biogenesis and photosystem assembly are strongly influenced by thylakoid membrane lipid composition and fluidity (McCourt et al., 1987; Jarvis et al., 2000; Routaboul et al., 2000; Babychuk et al., 2003), and, consequently, chilling-induced phase transition could be involved in the pale-green phenotype of young chilled leaves. Interestingly, in vitro experiments with artificial lipid membranes incorporated with vitamin E constituents have shown that both tocopherol and tocotrienol affect the structure and dynamics of membranes (Stillwell et al., 1992; Suzuki et al., 1993). Particularly, they disrupt molecular packing with gel state membranes, resulting in additional acyl chain motion. Therefore, the accumulation of high amounts of vitamin E in thylakoid membranes of HPPD-PDH plants could possibly affect membrane dynamics and physical properties and, hence, could mitigate chilling-induced impairment of chloroplast biogenesis.

Rather surprisingly, the tocotrienol level decreased substantially during long-term acclimation of HPPD-PDH leaves to high light at low temperature. One of the functions of the vitamin E constituents is to scavenge oxygen radicals (Fryer, 1992). During this process, the molecule donates its phenolic hydrogen atom to the oxygen radical and becomes a chromanoxyl radical. The latter radical can be reconverted to vitamin E by the reducing power of ascorbate (Munné-Bosch and Alegre, 2002) or by reaction with carotenoids (Böhm et al., 1997). It has been estimated that one α-tocopherol molecule is capable of protecting up to 220 molecules of polyunsaturated fatty acid before being consumed (Fukuzawa et al., 1982). During chilling stress in high light, α-tocopherol was found to be the first antioxidant that was degraded in cucumber (Cucumis sativus) leaves (Wise and Naylor, 1987). The loss of tocotrienols in stressed transgenic leaves thus could suggest either that they are less stable than...
tocopherols or that the strongly increased tocotrienol-carotenoid and tocotrienol-ascorbate ratios rendered the tocotrienol-recycling process less efficient. As a corollary, the consumption of tocotrienols suggests that, although no obvious symptom of photooxidation was found, a chronic production of ROS took place in young tobacco leaves growing under the stress conditions. Active scavenging of the produced ROS by tocotrienols may have participated to the acclimation of HPPD-PDH leaves to chilling stress in high light. Alternatively, the specific decrease in tocotrienol content could be explained by the fact that the promoter controlling the expression of the two transgenes, in contrast to endogenous tocopherol promoters, is not dependent on environmental conditions (Rippert et al., 2004).

To sum up, we have shown, for the first time in plants to our knowledge, that tocotrienols can protect membrane lipids from oxidation. Although this study was performed on plants that artificially accumulate high amounts of tocotrienols in leaf plastids, we assume that tocotrienols exert a similar function in other biological tissues, such as those in which tocotrienols usually accumulate (e.g. plant seeds). The antioxidative function of tocotrienols shown here in tobacco leaves confers an interesting agronomic trait to tocotrienol-accumulating plants. Moreover, tocotrienols have a number of pharmaceutical properties, ranging from the prevention of cholesterol accumulation to anti-proliferation action in cancer cells (Theriault et al., 1999). Consequently, tocotrienol-biofortified plants are potentially helpful, not only for agriculture in stressful environments but also for human health.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tobacco plants (Nicotiana tabacum var PR6) were grown on compost in a phytotron under controlled conditions of light (150 μmol photons m⁻² s⁻¹, 14 h d⁻¹) and temperature (25°C/20°C, day/night). Two transgenic lines (named HPPD-PDH 24-4 and HPPD-PDH 17-1) that coexpress the yeast (Saccharomyces cerevisiae) PDH gene and the Arabidopsis (Arabidopsis thaliana) HPPD gene were used in this study. This double transformation increases the HPP and homogentisate fluxes and causes a massive accumulation of tocotrienols (Rippert et al., 2004). Photooxidative stress was imposed by transferring plants to a growth chamber at 10°C with 300 μmol m⁻² s⁻¹ PFD for 14 h d⁻¹. Active scavenging of the produced ROS by tocotrienols may have participated to the acclimation of HPPD-PDH leaves to chilling stress in high light. Alternatively, the specific decrease in tocotrienol content could be explained by the fact that the promoter controlling the expression of the two transgenes, in contrast to endogenous tocopherol promoters, is not dependent on environmental conditions (Rippert et al., 2004).

Chlorophylls, Carotenoids, and Vitamin E

Photosynthetic pigments extracted from leaf discs in methanol were separated and quantified by HPLC, as reported previously (Havaux et al., 2004). Vitamin E was analyzed from freeze-dried leaves (approximately 150 mg) ground in liquid nitrogen and extracted three times with 2 mL of hexane in dim light in the presence of argon. The pooled supernatants were evaporated with argon and dissolved in methanol bubbled with argon. The methanolic extracts were stored at –80°C before analysis. Total lipids from chloroplast membrane fractions were extracted according to Folch et al. (1957), suspended in hexane, and centrifuged, and the resulting supernatant was dried under argon and dissolved in methanol. Tocophrers and tocotrienols were separated by HPLC as described elsewhere (Rippert et al., 2004). No internal standard was used during extraction. Consequently, since the vitamin E level was not adjusted for recovery, the data should be considered semi-quantitative.

Purification of Intact Tobacco Chloroplasts

Chloroplasts were purified from tobacco leaves according to Douce and Joyard (1982), with the following modifications. Crude chloroplasts were obtained from 400 to 500 g of young tobacco leaves and purified by isopycnic centrifugation on 50% preformed Percoll gradients (40,000 rpm for 55 min). Leaves were ground three times for 2 s, and the filtrate was centrifuged at 2,070 × g for 2 min. After resuspension, chloroplasts were loaded on top of the preformed Percoll gradients, and the gradients were centrifuged at 13,300 rpm for 10 min. Intact chloroplasts were collected from the gradients, diluted three to four times, and centrifuged at 2,070 × g for 2 min. All operations were carried out at 0°C to 5°C.

Purification of Plastoglobules, Envelope Membranes, and Thylakoid Membranes from Tobacco Chloroplasts

Purified intact chloroplasts were lysed in hypotonic medium in the presence of protease inhibitors (10 mg MOPS–NaOH, pH 7.8, 4 mg MgCl₂, 1 mg phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM ε-aminocaproic acid). Plastoglobules and envelope membranes were purified from the lysate by centrifugation at 70,000 × g for 4 h (SW41-Ti rotor; Beckman) on Suc gradients (0.93, 0.6, and 0.3 × Suc; Teysier et al., 1996). Plastoglobules were collected at the 0/0.3 × Suc interface. Envelope membranes were collected at the 0.6/0.93 × Suc interface, diluted three to four times in 10 mM MOPS–NaOH, pH 7.8, buffer containing protease inhibitors, and concentrated by centrifugation at 110,000 × g for 1 h. Thylakoids were collected at the bottom of the centrifuge tube of the Suc gradient. Chloroplast fractions were stored in liquid nitrogen in 10 mM MOPS–NaOH, pH 7.8, in the presence of protease inhibitors.

SDS-PAGE and Western-Blot Analyses of Chloroplast Fractions

SDS-PAGE analyses were performed as described by Chua (1980). For western-blot analyses, gels were transferred to a nitrocellulose membrane (BA85; Schleicher and Schuell). To analyze the purity of the membrane fractions, we used antibodies directed against the plastoglobule protein VTE1, the envelope protein ceQORH, and the thylakoid protein LHCIP. Western blots were revealed with ECL+ on a Typhoon 9400 phosphor imager (GE Healthcare) according to the manufacturer’s recommendations.

The procedure used for the western-blot analysis of antioxidant proteins (Prx Q, 2-Cys Prxs, and MsrA4) was described in detail in previous publications (Vieira Dos Santos et al., 2003; Rey et al., 2007; Collin et al., 2008). Briefly, tobacco leaves were ground in liquid nitrogen and the powder was resuspended in 50 mM MOPS–NaOH, 1 mM MgCl₂, 1 mM benzamidine, and 0.5 mM ε-aminocaproic acid. After centrifugation at 30,000 × g, 20 min, 4°C and precipitation of soluble proteins using acetone, the protein content was determined using a method based on bicinchoninic acid (BC Assay Reagent; Interchim). For western-blot analysis, polyclonal antibodies raised against Arabidopsis plastidic 2-Cys Prxs, poplar (Populus spp.) Prx Q, and poplar MsrA4 were used diluted 1:10,000, 1:1,000, and 1:1,000, respectively. The abundance of overoxidized 2-Cys Prx was investigated using a serum raised against Cys sulfenic and sulfonic acid forms of the protein (LabFrontier), as described by Rey et al. (2007).

Lipid Peroxidation

Lipid peroxidation was assessed by HPLC analysis of hydroxy fatty acids recovered from plant tissue after NaBH₄ reduction and saponification of total lipids. Leaves were frozen in liquid N₂ and stored at –20°C before extraction. Extraction was carried out according to the previously described procedure (Montillet et al., 2004). An aliquot of the extract (50 μL) was submitted to straight phase HPLC (Waters, Millipore) using a Zorbax Rx-SIL column (4.6 × 250 mm, 5-μm particle size; Hewlett-Packard), isocratic elution with 70:30:0.25 (v/v/v) hexane:diethyl ether:acetic acid at a flow rate of 1.5 mL min⁻¹, and UV detection at 234 nm. ROS-induced lipid peroxidation was evaluated from the levels of the different hydroxyoctadecatetraenoic acid (HOTE) isomers as described previously using 15-hydroxy-11,13(Z,E)icosadienoic acid as an internal standard (Montillet et al., 2004). Lipoxigenase-induced lipid peroxidation was estimated from the level of 13-HOTE after subtraction of racemic

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TL and Autoluminescence Imaging

TL measurements were performed with a custom-built apparatus that has been described (Havaux, 2003). The leaf samples (two discs of 8 mm in diameter) were slowly heated from 25°C to 150°C at a rate of 6°C min⁻¹. Leaf temperature was measured with a tiny K-type thermocouple, and luminescence emission was measured with a photomultiplier tube, the current of which was amplified by a transimpedance amplifier. Both leaf temperature and TL were recorded by a computer using a DaqPad-1200 data acquisition system (National Instruments). The TL band peaking at around 110°C (HTL2) corresponds to the thermal decomposition of lipid hydroperoxides yielding light-emitting triplet excited carbonyls (Devaraj et al., 1997; Vavilin and Ducruet, 1998; Havaux, 2003). The HTL1 band peaking at approximately 75°C is a pseudoband resulting from a competition between radiative thermolysis of peroxides and nonradiative hydrolysis below 100°C, which is dependent on leaf hydration (Ducruet and Vavilin, 1999).

Spontaneous photon emission associated with lipid peroxidation was also imaged at room temperature with a high-sensitivity, liquid nitrogen-cooled CCD camera, as described in detail elsewhere (Havaux et al., 2006). Plants were adapted to darkness for 2 h before recording photon emission. The integration time was 20 min, and on-CCD binning of 2 × 2 was used to increase detection sensitivity (the resulting resolution was 650 × 670 pixels).

Photosynthetic Electron Transport

Chlorophyll fluorescence emission from the upper surface of attached leaves was measured with a PAM-2000 fluorometer (Walz), as described previously (Havaux et al., 2003, 2005). The maximal quantum yield of PSII photochemistry was measured in dark-adapted samples by (Fm − Fo)/Fm = Fo/Fm' ratio, where Fm is the fluorescence level induced by a dim red light modulated at 600 Hz and Fo is the maximal level induced by an 800-ms pulse of intense white light. The quantum yield of PSII-mediated electron transport was measured in illuminated leaves by the ΔF/ΔF’ ratio, where ΔF' is the maximal fluorescence level and ΔF is the difference between Fm and the steady-state fluorescence level Fo. Leaves were illuminated with white light produced by a Oscht KL1500 light source equipped with a light guide.

Photosynthetic oxygen evolution by leaf discs (3.2 cm in diameter) was measured with a Clark-type oxygen electrode (Hansatech LD2/2), as described elsewhere (Walker, 1987). A carbonate/bicarbonate buffer was used to generate CO₂ inside the electrode. White light was produced by a Hansatech LS2 light source.

Carbohydrate Analyses

Soluble sugar (Glc, Fru, and saccharose) and starch contents of leaves were quantified as described. Five hundred milligrams of leaf tissue was ground three times in 0.5 M NaOH and centrifuged at 11,000g for 10 min. The cleared supernatants were pooled and neutralized to pH 7.5 with 6 M HCl and 1 M Tris-HCl (pH 7.5). An aliquot of 100 μL was used for saccharose hydrolysis by β-fructosidase in 100 mM acetate buffer (pH 4.6), and an aliquot of 200 μL was used for starch hydrolysis in 100 mM acetate buffer (pH 4.6) by amyloglucosidase at 35°C. Soluble sugars were quantified by a coupled enzymatic assay using hexokinase, phosphoglucoisomerase, and Glc-6-P dehydrogenase, by following the formation of NADPH at 340 nm (Bergmeyer and Benti, 1974).

Soluble Antioxidants

Ascorbate extracted from leaf discs in 4.5% metaphosphoric acid was measured by HPLC as described previously (Havaux et al., 2005). Reduction of ascorbate by 10 mM Tris-2-carboxyethylphosphine hydrochloride allowed total ascorbate content to be determined. Glutathione was measured using the HPLC method described elsewhere (Collin et al., 2008). Similar to total ascorbate, total glutathione was measured after Tris-2-carboxyethylphosphine hydrochloride treatment.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF047834 (Arabidopsis HPPD) and Z36035 (yeast PDH).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Separation of plastoglobules, envelopes, and thylakoids of wild-type chloroplasts by ultracentrifugation on a Suc gradient, and western blots of VTE1, CeQRH, and LHCP in each fraction.

Supplemental Figure S2. HPLC separation of the tocotrienol standards.

Supplemental Figure S3. Chromatograms of vitamin E, and vitamin E composition in plastoglobules purified from intact chloroplasts of old and senescing leaves of wild-type plants.

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Tocotrienols Function as Antioxidants in Tobacco Leaves