Constitutive overexpression of barley 4-hydroxyphenylpyruvate dioxygenase in tobacco results in elevation of the vitamin E content in seeds but not in leaves[1]

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Abstract With the aim to enhance the plant vitamin E content, the barley gene encoding 4-hydroxyphenylpyruvate dioxygenase was overexpressed in tobacco plants under control of the 35S promoter. Transgenic lines have a higher capacity for homogentisate biosynthesis as evident by a more than 10-fold higher resistance towards the bleaching herbicide sulcotrione. Seeds from transgenic lines have an up to two-fold enhanced level of vitamin E without a change in the ratio of γ-tocopherol and γ-tocotrienol. While the vitamin E content is not affected in leaves, the level of plastoquinone is enhanced in leaves of transgenic lines during leaf senescence.

Key words: 4-Hydroxyphenylpyruvate dioxygenase; Plastoquinone; Vitamin E; Tocopherol; Tobacco; Senescence

1. Introduction

Only plants and some cyanobacteria are able to synthesize the lipid soluble tocochromanols, better known as vitamin E [1]. Among the tocochromanols are the tocopherols, which possess a saturated phytyl side chain bound to a chromanol ring, and the tocotrienols, which have an unsaturated geranylgeranyl side chain. Based on the number and positions of methyl groups at the chromanol ring, four forms of tocopherols and tocotrienols, respectively, can be distinguished. In green leaves of higher plants the predominant form of vitamin E is α-tocopherol, whereas generally in seeds γ-tocopherol is the major form [1,2].

The best characterized function of vitamin E in mammals is to act as a free radical scavenger, thereby effectively inhibiting lipid oxidation. Epidemiological data suggest that high vitamin E intake correlates with a decreased risk of certain types of cancer and cardiovascular diseases [3]. Elevating the vitamin E content of oil seeds by genome-assisted strategies is of high interest for improving plant nutrient composition for human health [4].

The function of the different vitamin E forms in plants is far from being clear. Similarly as in animal cells, tocopherols act as antioxidants [5]. Recently, other possible functions of the tocochromanols in plants, like regulation of membrane fluidity and a role in intracellular signalling, have gained more attention [6,7].

The biosynthetic pathway for vitamin E has been elucidated several years ago [8], but the genes encoding the enzymes of the pathway have been identified only very recently (for a review see [4]). An important step in the early pathway is the formation of homogentisate (HGA) from 4-hydroxyphenylpyruvate and molecular oxygen, catalyzed by the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27). This step is common to the biosynthesis of both plastoquinone and vitamin E. Genes encoding HPPD have been identified from different plant species including *Daucus carota* [9], *Arabidopsis thaliana* [10] and *Hordeum vulgare* [11]. Fig. 1 shows the pathway for the vitamin E and plastoquinone biosynthesis as well as the catabolism of HGA leading to the formation of fumarate and acetoacetate [12,13]. By overexpression of the HPPD from *A. thaliana* in tobacco Garcia et al. [14] demonstrated that the HPPD is a cytosolic enzyme. A recent study reported a moderate increase in the tocopherol content of both seeds and leaves by homologous overexpression of the *hpd* gene in *A. thaliana* [15]. By condensation of HGA with prenyl chains the first true intermediates of vitamin E and plastoquinone are formed by specific polyprenyltransferases. Overexpression of the HGA phytyltransferase in *A. thaliana* under the control of a seed specific promoter resulted in an up to two-fold increase in tocopherol content of transgenic seeds [16].

A tocopherol cyclase catalyzing both the conversion of 2,3-dimethyl-5-phytyl-1,4-hydroquinone and 2,3-dimethyl-5-geranylgeranyl-1,4-hydroquinone to γ-tocopherol and γ-tocotrienol, respectively, has been identified in *A. thaliana* [6]. Seed specific overexpression of the γ-tocopherol-methyltransferase (γ-TMT), which catalyzes the final conversion of γ-tocopherol to α-tocopherol, altered the composition of tocopherols in *A. thaliana* seeds in favor of α-tocopherol, but did not change the total amount of tocopherols [17].

In this study we describe the effect of heterologous overexpression of a cDNA from *H. vulgare* encoding HPPD in tobacco. Transgenic plants were selected by their resistance towards the bleaching herbicide sulcotrione (Mikado). Seeds

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Abbreviations: HPPD, 4-hydroxyphenylpyruvate dioxygenase; HGA, homogentisate; γ-TMT, γ-tocopherol-methyltransferase

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from transgenic lines contain up to two-fold more vitamin E than the controls.

2. Material and methods

2.1. Transformation of tobacco plants and screening of transgenic lines

An *Eco*RI fragment covering the complete coding sequence of the HPPD cDNA of barley [11] was cloned blunt end into the *Sma*I cloning site of the plant binary vector pBinAR [18]. This site is located between the cauliflower mosaic virus 35S promoter and the octopine synthase polyadenylation signal.

The transformation construct was introduced into Agrobacterium strain GV3101 and used to transform leaf discs of tobacco (*Nicotiana tabacum*), cv. *Xanthi*, according to the method of [19]. Transformed cells were selected on regeneration medium containing 200 mg/l kanamycin.

Overexpression of the HPPD was tested by germination of seeds of transformed plants in microtiter plates in the presence of sulcotrione (Mikado). Seeds were incubated aseptically on blotting paper soaked in MS medium [20] with 0, 0.3, 1.5 or 3 μM sulcotrione in a growth chamber at 24°C and a daily light/dark regime of 16/8 h. After 7 days herbicide resistant seedlings were green and were chosen for further cultivation on soil.

2.2. Genomic Southern blot analysis

Total DNA was isolated from tobacco leaf tissue as described by [21]. Briefly, 100 mg leaf material was ground in 100 μl Microprep buffer (0.16 M sorbitol, 0.12 M Tris, pH 7.5, 22 mM EDTA, 0.8 M NaCl, 0.8% (w/v) CTAB, 0.8% (w/v) *N*-lauroylsarcosine), further diluted by addition of 550 μl Microprep buffer. After incubation at 65°C for 120 min, 750 μl chloroform:isoamyl alcohol (24:1) was added, followed by a centrifugation at 16000×*g* for 5 min. The aqueous phase was recovered and the DNA precipitated with 2-propanol at 16000×*g* for 5 min, washed with 70% (v/v) ethanol, and dissolved in 50 μl TE buffer.

30 μg DNA were digested overnight with the appropriate restriction enzymes. The resulting fragments were then separated by 0.8% (w/v) agarose gel electrophoresis. After neutral blotting to a Hybond N^+ membrane filter (Amersham Biosciences, Freiburg, Germany), hybridization was carried out with the 759 bp insert of the cDNA clone HvSD36 [22]. The probe was radioactively labelled with a 'HexaLabel DNA Labelling Kit' from MBI Fermentas (St. Leon-Roth, Germany).

2.3. RNA isolation and Northern blot analysis

Total RNA was isolated using the TRIzol reagent (Life Technology, Eggenstein, Germany) according to the manufacturer's protocol. PolyA$^+$-RNA was isolated from total RNA with a mRNA-isolation system (Promega, Mannheim, Germany). Northern analyses were performed with approximately 2 μg mRNA fractionated on a 1% (w/v) agarose gel containing 7.4% (v/v) formaldehyde and 3-(*N*-morpholino)propanesulfonic acid. Hybridization was carried out according to the manufacturer's instructions. The cDNA clone HvSD36, the EST clone cLET1G4 from tomato (1000 bp, accession AW040392) or a *rbcS* specific cDNA (800 bp) from soybean [23] were used as probes after radioactive labelling.

2.4. Extraction and analysis of prenyllipids

Vitamin E and plastoquinone were analyzed as described before [24]. Briefly, 100–200 mg of powdered frozen plant material was extracted with twice the volume of *n*-heptane/2-propanol (99.5+0.5) at −20°C for at least 24 h. After centrifugation at 15000×*g*, the clear supernatant was taken for analysis by HPLC. An extraction time of 24 h was shown to be sufficient for a complete extraction of vitamin E. During this extraction plastoquinone was completely oxidized.

In case of vitamin E, 20 μl of the sample was chromatographically analyzed using a LiChrosphere Si 100 (5 μm) column (10×250 mm) with *n*-heptane/2-propanol (99.5+0.5) as elutant and with a flow rate of 1.0 ml/min. Vitamin E forms were detected and quantified using a fluorescence detector (model RF10AXL, Shimadzu) set at $\lambda_{excitation}$ = 290 nm and $\lambda_{emission}$ = 328 nm. To calibrate the system and to verify the identity of individual peaks tocopherol and tocotrienol standards purchased from Merck (Darmstadt, Germany) were used.

For the analysis of plastoquinone, 20 μl of the same extract was analyzed using a LiChroSorb Si100 (5 μm) column (10×250 mm) as for vitamin E but with as elutant *n*-heptane/2-propanol (99.95+0.05) at a flow rate of 1 ml/min. Plastoquinone was detected by its absorption of ultraviolet light (λ = 254 nm).

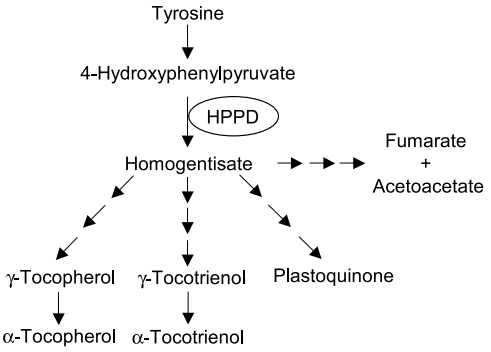

Fig. 1. Prenyllipids biosynthetic pathways and HGA catabolism.

3. Results

3.1. Selection and characterization of transgenic tobacco lines overexpressing barley HPPD

The HPPD is catalyzing the formation of HGA from 4-hydroxyphenylpyruvate and molecular oxygen (Fig. 1). HGA may either be used for the biosynthesis of tocopherols and tocotrienols or of plastoquinone. To enhance levels of HGA the complete cDNA encoding barley HPPD [11] was overexpressed in tobacco plants under control of the 35S CaMV promoter. Transgenic tobacco lines overexpressing barley HPPD were selected by their enhanced resistance towards the bleaching herbicide sulcotrione (Mikado), a competitive inhibitor of HPPD [25,14]. When seeds of homozygous lines were germinated in the wells of microtiter plates on MS-medium containing different concentrations of the herbicide, seedlings of transgenic lines were still green at Mikado concentrations of 3 μM (Fig. 2). In comparison, wild-type

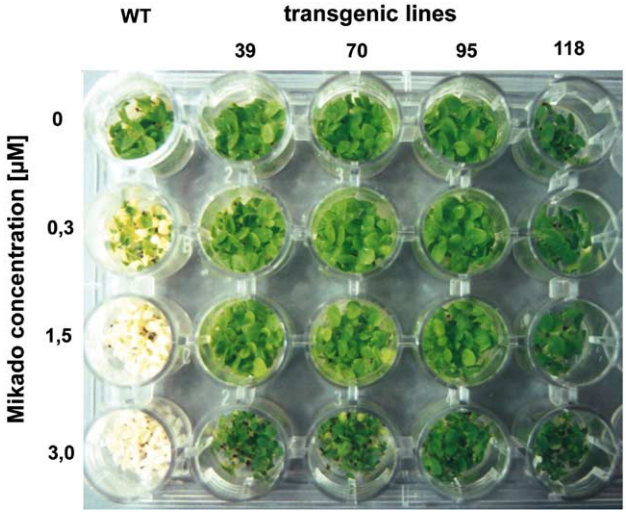

Fig. 2. Resistance of tobacco seedlings towards the herbicide sulcotrione. Seedlings were grown on MS medium supplemented with different concentrations of the herbicide (0.3, 1.5, 3.0 μM) in the wells of a microtiter plate. Two week old seedlings of transgenic lines 39, 70, 95 and 118 were compared with seedlings of the wild-type.

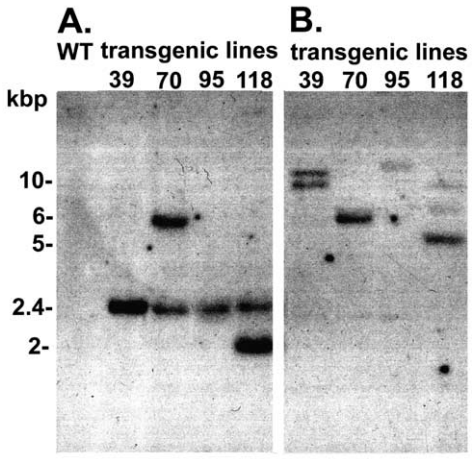

Fig. 3. Southern blot analyses. Total DNA derived from leaves of four transgenic lines and the tobacco wild-type was digested either with *Eco*RI and *Hin*dIII (A) or with *Eco*RI alone (B). 30 μg were applied to each lane, respectively. The filters were hybridized with a radioactively labelled 759 bp fragment derived from the barley *hpd* specific cDNA clone HvSD36 [22,11]. Positions of standard DNA fragments are given on the left.

seedlings were already bleached at a Mikado concentration of only 0.3 μM (Fig. 2).

Genomic Southern analysis was performed in order to examine the copy number of the barley *hpd* gene in the transgenic tobacco lines. The whole transformation construct can be excised by a double digest with *Eco*RI and *Hin*dIII. Digestion with *Eco*RI alone cuts the transformation construct only once in the region of the promoter. Independent transformation lines generated *Eco*RI fragments of different lengths. The double digest with *Eco*RI and *Hin*dIII yielded a fragment of 2.4 kbp in all four lines (Fig. 3A). Additional signals were detected in lines 70 and 118. In case of line 70 this signal may result from an incomplete restriction of the DNA by *Hin*dIII since the same signal occurred when DNA was digested with *Eco*RI only (Fig. 3B). The 2 kbp signal in case of line 118 may represent a truncated transformation construct. Despite the additional signals in line 70 and 118, all four lines contain the intact transformation cassette including the 35S promoter and the barley *hpd* gene.

Hybridization of total DNA restricted with *Eco*RI showed two signals in case of line 39, indicating that this line contains at least two copies of the barley *hpd* gene. In comparison, lines 70 and 95 contain one copy and line 118 probably three copies of the gene (Fig. 3B). Presumably, one of these copies of the barley *hpd* gene in tobacco line 118 is truncated.

In order to investigate whether the barley transgene is expressed in tobacco leaves, a Northern blot analysis with poly-A$^+$-RNA was performed. Hybridization with the barley *hpd* probe revealed that the transgene is expressed in all four transgenic lines (Fig. 4A). The barley *hpd* probe did not cross-react with the tobacco *hpd* transcript under the conditions used in the experiment, since no signal was obtained with polyA$^+$-RNA prepared from the wild-type. Subsequently, the same filter was hybridized with the EST clone cLET1G4 (accession no. AW040392) from tomato. cLET1G4 is specific for the *hpd* gene and is hybridizing with the tobacco *hpd* gene, the sequence of which is still unknown. The cLET1G4 did not crossreact with the barley *hpd* gene (data not shown). This hybridization showed that the tobacco *hpd* transcript is detectable in all four transgenic lines as well as in the wild-type. The weak signals in case of transgenic line 70 are due to unequal loading as evident by a control hybridization of the same filter with the *rbcS* specific probe from soybean [23].

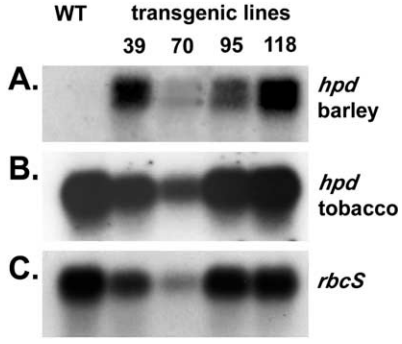

Fig. 4. Northern blot analyses with polyA$^+$-RNA. RNA was prepared from mature leaves of the four transgenic lines 39, 70, 95 and 118 and the wild-type. The same filter was subsequently hybridized with radioactively labelled probes specific for the barley *hpd* gene (A), the tobacco *hpd* gene (B) and the tobacco *rbcS* gene (C). Whereas in case of the barley *hpd* gene the barley cDNA HvSD36 (see Fig. 3) has been used as probe, the tobacco genes have been detected by heterologous probes derived from tomato (*hpd*) and soybean (*rbc*S).

3.2. HPLC analyses of tocopherols and plastoquinone-9 in tobacco leaves

In order to investigate whether overexpression of the barley *hpd* gene in tobacco coincides with an enhanced level of to-

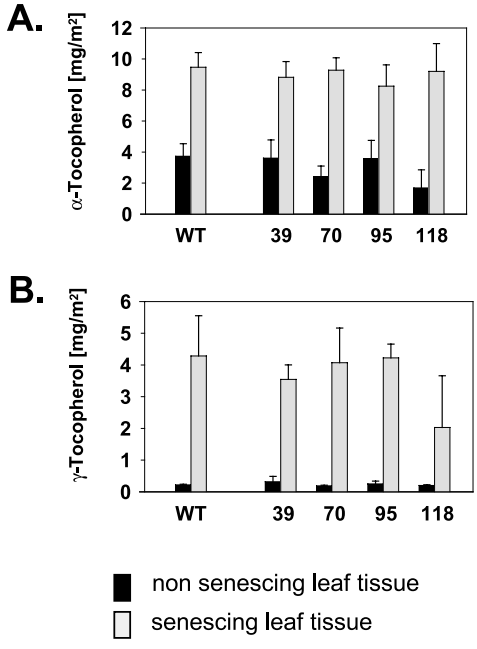

Fig. 5. Tocopherol content of tobacco leaves. Prenyllipids were extracted from mature (black columns) and senescing leaves (grey columns) of tobacco wild-type and transgenic lines 39, 70, 95 and 118. Senescing leaves showed a decrease of 50% in chlorophyll content. HPLC analyses were performed with leaf extracts from four individual plants, respectively. Levels of α-tocopherol (A) and γ-tocopherol (B) are based on leaf area.

copherols in leaves, extracts from mature leaves were analyzed for their tocopherol content by HPLC. Plants were grown in growth chambers under well controlled conditions. These precautions were absolutely necessary to avoid the influences of developmental stage, water availability, light intensity, and diurnal changes on the vitamin E content of the individual plants. With regard to their tocopherol content and composition, no differences could be observed between wild-type and transgenic plants. It is well known that tocopherols accumulate in leaves during senescence [26,27]. This is in contrast to the observation of Tanaka et al. [28], who could not observe an increase in tocopherol content in senescing tobacco leaves under the growth conditions used. However, when we analyzed senescing leaves, whose chlorophyll content was decreased by 50%, for their tocopherol content, it was observed that they also have a higher tocopherol content compared to non-senescing leaves. While in senescing leaves the level of α-tocopherol increased between two- to three-fold, the level of γ-tocopherol increased up to 10-fold (Fig. 5A,B). When the tocopherol content in senescing leaves from wild-type and transgenic plants was compared, no significant differences, neither in α-tocopherol content nor in the content of γ-tocopherol, could be detected (Fig. 5A,B).

As shown in Fig. 1, HGA is the aromatic precursor of plastoquinone-9, too. To investigate whether the enhanced capacity to synthesize HGA, results in a higher plastoquinone-9 content in leaves of the transgenic tobacco lines, leaf extracts were analyzed for their plastoquinone-9 content by HPLC. While there is no difference between wild-type and transgenic lines with regard to the plastoquinone-9 content in non-senescing leaves, plastoquinone-9 levels are significantly enhanced in senescing leaves of transgenic lines 70 and 118 compared to the wild-type (Fig. 6). However, this higher plastoquinone-9 content could only be seen in senescing leaves when the chlorophyll content was decreased by more than 60%. The increased plastoquinone content had no effect on the maximum efficiency of photosystem II in transgenic lines 70 and 118 (data not shown).

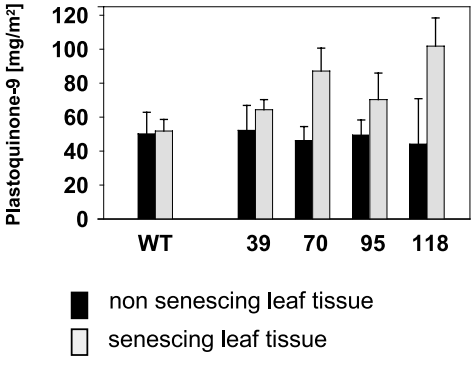

Fig. 6. Plastoquinone-9 content of tobacco leaves. Prenyllipids were extracted from mature (black columns) and senescing leaves (grey columns) of tobacco wild-type and transgenic lines 39, 70, 95 and 118. Senescing leaves showed a decrease of more than 60% in chlorophyll content. HPLC analyses were performed with leaf extracts from four individual plants, respectively. Levels of plastoquinone-9 are based on leaf area.

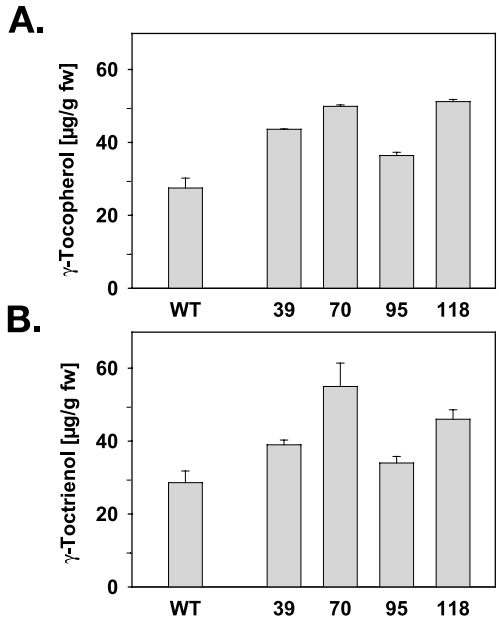

Fig. 7. γ-Tocopherol and γ-tocotrienol contents of tobacco seeds. Levels of tocochromanols were determined by HPLC analyses (three replicates) of extracts derived from three individual plants. Levels of γ-tocopherol (A) and γ-tocotrienol (B) are based on fresh weight.

3.3. HPLC analyses of tocopherols and tocotrienols in tobacco seeds

Seeds of homozygous transgenic lines and seeds from wild-type plants were analyzed for their vitamin E content by HPLC. The predominant forms of vitamin E in tobacco seeds were γ-tocopherol and γ-tocotrienol, which occur in almost equal amounts. Other tocopherol and tocotrienol forms were hardly detectable. Compared to seeds from wild-type plants, seeds from transgenic lines showed an up to twofold higher content of both forms, while the vitamin E composition remained unchanged (Fig. 7).

Both, tocochromanols in seeds and plastoquinone-9 in senescing leaves accumulated to the highest levels in transgenic lines 70 and 118 indicating that production of HGA is highest in these two lines.

4. Discussion

By constitutive overexpression of the barley *hpd* gene under control of the 35S promoter, in this study plants with an enhanced capacity for the biosynthesis of HGA were obtained. The higher biosynthetic capacity is obvious by a higher resistance towards the bleaching herbicide sulcotrione. Since the transformation was performed with the 35S promoter, it was expected that HGA synthesis was enhanced in all plant tissues. Nevertheless, tocochromanol content was elevated only in seeds and not in leaves. However, an increase of the seed tocochromanol content of 11% was achieved by Tsegaye et al. [15] by homologous overexpression of the *hpd* gene under control of the 35S promoter. When a seed specific promoter was used instead, an up to 28% increase in seed tocopherol content was obtained. In contrast to our study, overexpression of the *hpd* gene in *A. thaliana* resulted also in an increase in the tocochromal content of leaves.

The differences between the two studies may be due to

differences between the two plants used for overexpression. It is interesting that homologous overexpression of the *hpd* gene is at least in *A. thaliana* suited to enhance the tocochromal content of leaves while heterologous expression of the *hpd* gene seems to be better suited to enhance the tocochromanol level of seeds.

Analyses of plastoquinone showed that in senescing leaves the surplus of HGA is at least partially used for the synthesis of plastoquinone. This result is rather surprising, considering that the photosynthetic apparatus is degraded during senescence [29]. In mature leaves the surplus of HGA is neither used for the synthesis of tocopherols nor of plastoquinone. It is possible that in non-senescing leaves HGA is degraded via fumarate and acetoacetate. Indeed, preliminary enzymatic measurements indicate an increased activity of the HGA dioxygenase in the transgenic tobacco leaves (data not shown). This observation could also account for the unaltered transcript level of the endogenous *hpd* in tobacco. Otherwise one might expect that an increased HGA pool would downregulate the expression of the endogenous *hpd* through a negative feedback mechanism. So far, only the senescence promoting growth regulators ethylene and jasmonate as well as oxidative stress could be shown to induce the expression of the *hpd* [11]. In this respect, it is of interest that a tyrosine aminotransferase, which catalyzes the transamination from tyrosine to 4-hydroxyphenylpyruvate, is also induced by jasmonate [30].

An increase in tocopherol content during leaf senescence as shown here for tobacco has previously been demonstrated with barley flag leaves [27]. An accumulation of tocopherol in senescing leaves has been assigned to a higher demand of antioxidative compounds in the senescing leaves. In barley leaves as well as in tobacco leaves the increase in γ-tocopherol content is much more pronounced than the increase in α-tocopherol content. This suggests that the activity of the γ-TMT declines with senescence.

Besides a limited capacity to accumulate tocopherols, limitations in other steps of the biosynthesis pathway could be responsible for this restriction in the capability to accumulate higher levels of tocopherols. In this study sense expression of the barley *hpd* gene under control of the 35S promoter resulted in an up to two-fold increase in tocochromanol content of tobacco seeds. A similar increase has been obtained by sense expression of the gene encoding HPT from *Synechocystis* PCC6803 under the control of the seed specific napin promoter [16]. Taken together the results from both studies suggest that one of the enzymatic steps following the formation of HGA is rate-limiting for tocochromanol biosynthesis. Earlier studies suggested that the biosynthesis of geranylgeranylpyrophosphate may be limiting for tocopherol biosynthesis [31]. Accordingly, a reduction in geranylgeranyl reductase activity by antisense expression in tobacco plants resulted in a partial reduction of the tocopherol content [28,32]. In these tobacco plants, similar as in the *A. thaliana* mutant *vte*1 lacking tocopherols, due to impairment of the tocopherol cyclase gene [6], photosynthesis was only affected under conditions of oxidative stress in high light.

In contrast to the results obtained by [16] with seeds of *A. thaliana* plants transformed with the gene encoding HPT1, in seeds of tobacco transformed with the barley *hpd* gene, the ratio of the two predominant tocochromanol forms is unaffected. Since in *A. thaliana* α-tocopherol did not increase likewise as γ-tocopherol it has been suggested that the activity of the γ-TMT is saturated at the wild-type level [16]. In tobacco seeds almost exclusively γ-tocopherol is found besides γ-tocotrienol. This suggest that the γ-TMT is not active in tobacco seeds.

Recent investigations suggest that not only α-tocopherol but also the other vitamin E forms play an important role for human health. Obviously, the different vitamin E derivatives fulfil specific functions in humans, apart from being antioxidants [33,34]. It will be of interest to unravel the function of γ-tocopherol and tocotrienols in seeds and in general.

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