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Reduced Activity of Geranylgeranyl Reductase Leads to Loss of Chlorophyll and Tocopherol and to Partially Geranylgeranylated Chlorophyll in Transgenic Tobacco Plants Expressing Antisense RNA for Geranylgeranyl Reductase[1]

Ryouichi Tanaka[2], Ulrike Oster, Elisabeth Kruse, Wolfhart Rüdiger, and Bernhard Grimm*

Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, 06466 Gatersleben, Germany (R.T., E.K., B.G.); and Botanisches Institut der Ludwig Maximilian Universität München, Menzinger Strasse 67, 80638 München, Germany (U.O., W.R.)

The enzyme geranylgeranyl reductase (CHL P) catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate. We identified a tobacco (*Nicotiana tabacum*) cDNA sequence encoding a 52-kD precursor protein homologous to the Arabidopsis and bacterial CHL P. The effects of deficient CHL P activity on chlorophyll (Chl) and tocopherol contents were studied in transgenic plants expressing antisense CHL P RNA. Transformants with gradually reduced *Chl P* expression showed a delayed growth rate and a pale or variegated phenotype. Transformants grown in high (500 μmol m^{-2} s^{-1}; HL) and low (70 μmol photon m^{-2} s^{-1}; LL) light displayed a similar degree of reduced tocopherol content during leaf development, although growth of wild-type plants in HL conditions led to up to a 2-fold increase in tocopherol content. The total Chl content was more rapidly reduced during HL than LL conditions. Up to 58% of the Chl content was esterified with geranylgeraniol instead of phytol under LL conditions. Our results indicate that CHL P provides phytol for both tocopherol and Chl synthesis. The transformants are a valuable model with which to investigate the adaptation of plants with modified tocopherol levels against deleterious environmental conditions.

The main constituents of the photosynthetic apparatus are Chls, carotenoids, the plastid-encoded apoproteins of the core complex of the reaction centers, and the nuclear-encoded light-harvesting Chl-binding proteins of the antenna complexes. Control of pigment metabolism and expression of pigment-binding proteins ensure a synchronous synthesis of all components in stoichiometric amounts to prevent pigment or protein degradation or photooxidative deterioration.

Chl consists of two moieties, chlorophyllide and phytol, which are formed from the precursor molecules 5-aminolevulinate and isopentenyl diphosphate, respectively, in the two different pathways of tetrapyrrole and isoprenoid biosynthesis. Most of the previous investigations of Chl biosynthesis have emphasized the tetrapyrrolic pathway, which is entirely located in plastids and converts Glu to Chl. Most of the genes involved in tetrapyrrole biosynthesis have been characterized previously (Chadwick and Ackrill, 1994; von Wettstein et al., 1995; Porra, 1997; Rüdiger, 1997; Grimm, 1998).

The branched isoprenoid pathway is rather complex and comprises enzymatic steps in at least two compartments. The cytosolic isoprenoid-synthesizing pathway proceeds from acetyl-CoA via mevalonate in the plant cytoplasm, leading, for example, to sterol compounds. Incorporation studies of labeled early precursors indicated a mevalonate-independent pathway in plastids for phytol biosynthesis, for which 1-deoxy-xylulose-5-P is an intermediate (Rohmer et al., 1993; Lichtenthaler et al., 1997). The exact localization of the pathways for carotenoids and other end products was hampered to a certain extent because isopentenyl diphosphate, which is the common intermediate of both pathways, is transferred through the plastid envelope (Kreuz and Kleinig, 1984; Gray, 1987).

Four molecules of isopentenyl diphosphate are subsequently joined to form the C_{20}-intermediate GGPP, which is then allocated to the synthesis of various end products such as carotenoids, quinones, Chl, or tocopherol. The hydrogenation of GGPP is catalyzed by a CHL P (EC 1.3.1.-). The enzyme Chl synthase links tetrapyrrole and isoprenoid metabolism by esterifying chlorophyllide with GGPP or PhyPP. In etiolated plants Chl synthase esterifies chlorophyllide preferentially with GGPP to form Chl a_{GG}. The recombinant Chl synthase that is encoded in the G4 gene of Arabidopsis (Gaubier et al., 1995) and overexpressed in *Escherichia coli* also gives preference to GGPP relative to PhyPP (Oster and Rüdiger, 1997). The NADPH-dependent hydrogenation to the phytol chain of Chl is observed after prenylation of chlorophyllide (Schoch et al., 1977; Benz et al., 1980).

Conversely, Chl synthase assayed from green plants favors PhyPP for Chl *a* synthesis rather than GGPP (Soll et al., 1983; Rüdiger, 1987). PhyPP is the preferential substrate

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[2] Present address: The Institute of Low Temperature Science, Hokkaido University, N19 W8, Sapporo 060–0819, Japan.

* Corresponding author; e-mail grimm@ipk-gatersleben.de; fax 49–39482–5139.

Abbreviations: Chl, chlorophyll; CHL P, geranylgeranyl reductase; GGPP, geranylgeranyl diphosphate; HL, high light; LL, low light; PhyPP, phytyl diphosphate.

of two overexpressed proteins, the Chl synthase derived from the *chlG* gene of *Synechocystis* and the bacteriochlorophyll synthase encoded by the *Rhodobacter bchG* gene (Oster et al., 1997).

PhyPP is also an obligatory precursor for the synthesis of tocopherol (Soll and Schultz, 1981), and is directed into the tocopherol-synthesizing pathway by condensation with homogentisate derived from the shikimate pathway. Several methylations and a cyclization step of the quinol intermediate leads to α-tocopherol, the major form of the vitamin E fraction (Soll, 1987). Tocopherol prevents the Chl-photosensitized oxidation of thylakoid components, especially when plants are subjected to environmental stress, mainly by quenching activated singlet oxygen or by scavenging oxygen radicals (Fryer, 1992).

A *bchP* gene has been previously detected as part of the 46-kb photosynthetic gene cluster of *Rhodobacter capsulatus* (Marrs, 1981; Zsebo and Hearst, 1984) encoding almost all constituents required for photosynthesis. Insertional mutagenesis of the *bchP* gene resulted in a mutant that produced bacteriochlorophyll esterified with geranylgeraniol instead of phytol (Bollivar et al., 1994). The *bchP* gene encodes CHL P. Electron transfer and energy transfer from the light-harvesting complex to the reaction center were apparently not affected in the *R. capsulatus* mutant. Nevertheless, the growth rate under photosynthetic conditions was severely reduced in the mutant (Bollivar et al., 1994). The authors suggested a reduced stability of the pigment-protein complexes, if bacteriochlorophyll is esterified with geranylgeraniol. Other reasons for a reduced growth rate could not be ruled out. Nevertheless, all normal functions were restored when the homologous *chlP* gene from *Synechocystis* sp. PCC 6803 was expressed in the *Rhodobacter bchP*-deficient mutant (Addlesee et al., 1996).

We isolated the tobacco *Chl P* sequence encoding CHL P and examined its metabolic function as well as its expression in transgenic tobacco plants. CHL P is located at the branch point toward Chl and tocopherol. Our aim was to improve the understanding of the molecular and physiological effects of reduced synthesis of PhyPP on the controlled distribution of substrate for Chl and tocopherol synthesis under different light conditions.

MATERIALS AND METHODS

Plant Growth and Harvest

Wild-type (*Nicotiana tabacum* var Samsun NN) and transgenic tobacco plants were cultivated in growth chambers with a 16-h light/8-h dark cycle at 25°C. The light intensities were 500 μmol m^{-2} s^{-1} (HL) or 70 μmol m^{-2} s^{-1} (LL). Primary transformants were used for analysis. Leaves were harvested from 8-week-old plants grown in growth chambers, frozen in liquid nitrogen, and analyzed immediately, freeze-dried, or stored at −80°C before analysis.

Isolation and Analysis of a cDNA Clone Encoding CHL P Protein

A tobacco cDNA library (*Nicotiana tabacum* SR1, Stratagene) in a Lambda ZAP II cDNA library was screened using the expressed sequence tag clone 4D9T7P (accession no. T04791) from Arabidopsis obtained from the Arabidopsis Biological Research Center (Ohio State University, Columbus). The sequence is homologous to that of the *bchP* gene of *Rhodobacter capsulatus* (Young et al., 1989) and of Arabidopsis *Chl P*. The cDNA sequences were analyzed with the PCGENE program (Intelligenetics, Mountain View, CA). Alignment of peptide sequences was done with the Clustal W program.

Construction of a *Chl P* Antisense Gene and Plant Transformation

The full-length cDNA sequence was cut out of the vector with the restriction enzymes *Kpn*I and *Xba*I and ligated into the same restriction sites of the plant binary vector BinAR (Höfgen and Willmitzer, 1992), a pBIB derivative containing the cauliflower mosaic virus 35*S* promoter. The transformation of tobacco leaf discs was mediated by *Agrobacterium tumefaciens*, as described by Horsch et al. (1985). The insertion of copies of the transgene was confirmed by kanamycin resistance of regenerated explants and by genomic Southern hybridization or PCR amplification using a *Chl P*-specific probe and oligonucleotide primers.

RNA Analysis

Total RNA was isolated by the acid-phenol extraction method (Chomczinski and Sacchi, 1987). Aliquots of 10 μg of RNA were blotted onto nylon membranes (Hybond N, Amersham) and hybridized with [^{32}P]dCTP using the nick-translation method. Hybridized filters were exposed to radiographic film (Kodak) or to phosphor-imaging plates (Fuji Film, Tokyo) and analyzed (STORM 960, Molecular Dynamics, Krefeld, Germany). Equal loading of samples was controlled by rehybidizing the RNA filter with a cDNA probe for 18*S* rRNA (Thompson et al., 1994).

Antiserum Preparation and Western Analysis

Two oligonucleotide primers were designed to amplify the coding sequences of *Chl P*: Csyn1, 5′cgc cat ggg ccg caa tct tcg tgt tgc ggt 3′; Csyn 2, 5′gca gat ctg tcc att tcc ctt ctt agt gca 3′. The PCR fragment was cloned into the *Nco*I and *Bgl*I sites of the expression vector pQE 60 (Qiagen, Hilden, Germany). The subcloned tobacco *Chl P* sequence continues behind the initiation codon of the expression plasmid with the nucleotide at position 148 of the cDNA clone (accession no. AJ007789). Overexpression of recombinant CHL P protein was performed in *Escherichia coli* XL-1 Blue or SG 13009 (Stratagene). The protein was purified by metal chelate affinity chromatography and used for immunization of rabbits. Antiserum was collected after triple injection of the antigen.

Plant material (100 mg) was ground under liquid nitrogen, suspended in 1 mL of solubilization buffer (56 mM Na_2CO_3, 56 mM DTT, 2% SDS, 12% Suc, and 2 mM EDTA), and denatured for 15 min at 70°C. The soluble protein fraction was quantified and 10-μg protein aliquots were

analyzed by western blot with the anti-CHL P antiserum using an immunoblotting kit (ECL, Amersham).

Analysis of Chl

Leaf tissue (100 mg fresh weight) was pulverized in liquid nitrogen with a mortar and pestle and extracted twice with 400 μL of acetone:water (3:1, v/v). The liquid phases were collected in a 2-mL test tube. The extraction was repeated three times until the pellet was colorless. The combined acetone extracts were cleared by centrifugation and mixed with 500 μL of *n*-hexane. The hexane phase was separated and the acetone phase again extracted with the same volume of *n*-hexane. A small aliquot of the combined hexane phases was washed with water until it was free of acetone. A_{660} and A_{642} were determined after a suitable dilution (normally 1:10) in a spectrophotometer (model 8451A, Hewlett-Packard) and used for calculation of the total contents of Chl *a* and *b* according to the method of French (1960). The rest of the combined hexane phases was acidified with three drops of concentrated HCl. The color of the solution changed from green to brown, indicating the formation of pheophytin from Chl.

The hexane phase was then repeatedly washed with water until the aqueous phase reached a pH of $\geq$5.5. The last traces of water were removed from the hexane phase by freezing at −20°C. Hexane was then removed by evaporation and the precipitate was dissolved in 500 μL of acetone. Twenty microliters of the 1:10 diluted samples was applied for HPLC analysis (model 480, Gynkothek, Ramsey, NJ). Pigments were separated on a column (4 × 250 mm) filled with RP18 (Gromsil 120, Grom Analytic, Herrenberg, Germany) at 1.2 mL/min with the following gradient consisting of 60% acetone (solvent A) and 100% acetone (solvent B): 75% A/25% B for 2 min, followed by 45% A/55% B for 2 min, 30% A/70% B for 11 min, and 100% B for 8 min. Pigments were detected by a UV-visible light detector (model SP5V, Shimadzu, Columbia, MD) at 410 nm and by a fluorescence detector (model RF551, Shimadzu) at 665 nm_{em} and 425 nm_{ex}. The peak areas indicated the ratios of Chl a_{GG} to Chl a_{Phy} and Chl b_{GG} to Chl b_{Phy}.

Analysis of Tocopherol

Tocopherol was measured independently in both laboratories by two methods. In the first, leaf material was pulverized in liquid nitrogen and lyophilized. A precisely weighted 5-mg aliquot of the dry powder was extracted four times in a precooled mortar with 350 μL each of dioxane:*n*-hexane (1:1, v/v), and the combined supernatants were cleared by centrifugation and evaporated. The residue was dissolved in 100 μL of dioxane:*n*-hexane (3:97, v/v). For each analysis, 20 μL of this solution was analyzed by HPLC (model 300C, Gynkothek) using a column (4.6 × 250 mm) filled with Nucleosil 50 (5 μm) at a flow rate of 1.5 mL/min with dioxane:*n*-hexane (3:97, v/v). Tocopherol was quantified at 295 nm_{ex} and 325 nm_{em} using a fluorescence detector (model RF1001, Shimadzu).

In the second method, tocopherol was extracted from frozen leaf powder with acetone containing 10 μM KOH and separated on a HPLC system equipped with a C_{18} column (3.9 × 150 mm, Nova-Pak, Waters) with a gradient of solvent A (30% methanol, and 10% 0.1 M ammonium acetate, pH 5.2) and solvent B (100% methanol) as follows: a linear gradient from 6% A/94% B at 0 min to 1% A/99% B at 10 min until 23 min with the same ratio of solutions A and B. Standards for α, β/γ, and δ tocopherol and for α, β/γ, and δ tocotrienol were purchased from Merck (Darmstadt, Germany) and used to quantify and qualify the tocopherol forms eluted by our HPLC program.

Analysis of Carotenoids

Carotenoids were extracted from 100 mg of leaf powder with acetone containing 10 μM KOH and separated by HPLC with a linear gradient beginning with 100% eluate A (86.7% acetonitrile, 9.6% methanol, and 3.6% 0.1 M Tris-HCl, pH 8.0) to 100% eluate B at 15 min (80% methanol and 20% hexane) on a 5-μm column (Lichrosphere 100 RP-18, Merck, Darmstadt, Germany) and monitored by a photodiode array detector (model 996, Waters) at a flow rate of 1 mL/min. Carotenoid standards were purchased from Roth (Karlsruhe, Germany).

RESULTS

Tobacco Chl P cDNA Sequence Encoding CHL P and the Expression of Recombinant Protein

A full-length cDNA clone encoding CHL P was identified. The cDNA sequence is composed of 1510 nucleotides without the poly(A^+) chain and is deposited in the database under accession no. AJ007789. Nucleotides 1 through 1392 encode a 52-kD protein consisting of 464 amino acid residues. The deduced peptide sequence shows similarity to the *Mesembryanthemum crystallinum* CHL P (accession no. AF069318) (82% identical amino acid residues), to the Arabidopsis CHL P sequence (accession no. Y14044) (81% identical amino acid residues) (Keller et al., 1998), to the *Synechocystis* sp PCC 6803 ChlP (accession no. Q55087) (67%) (Addlesee et al., 1996), and to the *R. capsulatus* counterpart (34%) (Zsebo and Hearst, 1984; Bollivar et al., 1994). In contrast to the bacterial peptides, the three plant sequences of CHL P contain amino-terminal extensions that resemble plastid transit sequences. The overall similarity among the five sequences was 29.1%.

The coding region of a truncated CHL P peptide (amino acid residues 50–464) was fused in frame behind the initiation codon into an *E. coli* expression vector. The beginning of the open reading frame codes for the Met-Gly-Arg-Asn-Leu of the recombinant CHL P. The recombinant protein was insoluble in aqueous solution. The His-tagged protein was dissolved in 8 M urea and was purified by metal chelate affinity chromatography as recommended by manufacturer's instructions. The purified protein of an approximate molecular mass of 47 kD was injected into rabbits for immunization.

Phenotypical Differences between Transgenic Plants Expressing Antisense-Oriented *Chl P* Genes and Control Plants

The full-length *Chl P*-cDNA sequence was inserted in inverse orientation between the cauliflower mosaic virus 35*S* promoter and the 3′ termination sequence of the octopine synthase gene of the binary plant vector BinAR. The antisense gene construct was introduced into the tobacco genome by *A. tumefaciens*-mediated transformation. Approximately 100 different transgenic lines were generated and analyzed for the insertion of copies of the transgene.

The transformants were generally characterized by a growth rate slower than or similar to that of control plants and a gradually reduced Chl content compared with the wild type. Most of the transgenic lines displayed a uniform low pigmentation, and some of them had yellow areas along the leaf veins or different variegation patterns (Fig. 1). The green pigmentation was generally more reduced in older than in younger leaves of the same transformant. The transformants grown in the greenhouse or in the growth chamber under controlled conditions did not show any necrotic leaf lesions that could be generated by accumulating photosensitizing tetrapyrrole intermediates.

Metabolic Effects of Reduced Expression of *Chl P* in Transgenic Plants

At first, all transformants were phenomenologically and biochemically evaluated to select a few transgenic lines for further detailed analysis. The primary data were obtained with plants grown in the greenhouse under ambient conditions. The plants were exposed to diurnal changes in temperature (15°C–24°C) and light intensity (up to 800 μM m^{-2} s^{-1}) before analysis. Table I illustrates the gradual variation of the inhibitory effects on Chl and tocopherol contents by *Chl P* antisense RNA expression among a representative set of transformants (lines 6, 10, 20, 21, 24, and 47) and wild-type plants. These lines represent a broad range of gradually increasing transgenic phenotypes; they retained these characteristics in the course of the studies over almost 2 years and were characterized by a progressive reduction in growth rate and pigmentation. Their progenies of the T_1 and T_2 generation showed the same typical deficiency symptoms or were more severely impaired in growth and pigmentation.

Figure 1. Primary transformant 6 with *Chl P* antisense genes (PL24–6) and a wild-type tobacco plant (SNN). Plants were grown for 8 weeks under greenhouse conditions in an average light intensity of 300 μmol m^{-2} s^{-1}.

Table I. *Comparative analysis of Chl P antisense RNA-expressing primary transformants and control plants*

The fifth leaf of each plant was harvested and analyzed. Total Chl, Chl_{GG}, Chl_{Phy}, and tocopherol were determined by HPLC and are given as percentage of the wild-type (WT) levels (except for Chl_{GG}, which is given as percentage of the total Chl in the respective transformant).

Plant	Chl_{GG}	Total Chl	Chl_{Phy}	Tocopherol
	%		% *WT*	
WT	0	100	100	100
6	53	32	15	14
10	56	40	18	19
20	7	59	55	52
21	4	90	87	99
24	30	99	70	91
47	25	37	28	18

The pale-green leaves of some transgenic plants correlated with the reduced total Chl content. The severely affected lines 6, 47, and 10 accumulated only 32%, 37%, and 40%, respectively, of the wild-type Chl content. The transformants also contained a lower tocopherol content. Lines 6, 47, and 10 also showed the strongest deficiency of tocopherol (14%, 18%, and 19% of the control values, respectively). As indicated, other transgenic lines displayed only minor decreases in Chl and tocopherol contents. Tocotrienol could be an expected product if GGPP were also used for the synthesis of tocopherol. The HPLC elution program allowed the separation of tocopherol and tocotrienol derivatives, but tocotrienol was not detected in extracts of the transformants.

The esterification of Chl with various alcohols was analyzed by HPLC. To avoid allomerization and oxidation, we removed the central Mg^{2+} and analyzed the corresponding pheophytins. A typical chromatogram is shown in Figure 2. In the control samples, pheophytins *a* and *b* (Phe α_{Phy} and Phe b_{Phy}) were detectable as the only Chl derivatives. In the samples of the transformants, three new peaks were identified by co-chromatography with authentic samples: the new compound Phe a'_{Phy} and Phe α and Phe *b* esterified with geranylgeraniol (Phe a_{GG} and Phe b_{GG}). The identity of the pigments was confirmed by the absorption and the fluorescence emission spectra of the single peaks and by comparison with the authentic compounds. We could not detect in any of the transformants the pigments containing intermediate alcohols between geranylgeraniol and phytol. This observation of the steady-state content in transgenic plants differs from the results of the in vitro assays with recombinant Arabidopsis CHL P (Keller et al., 1998). The small peaks between those of the identified pigments do not indicate Chl derivatives, but they exhibited only diffuse fluorescence and absorption spectra (Fig. 2).

The percentages of Chl a_{GG}, Chl b_{GG}, Chl a_{Phy}, and Chl b_{Phy} were determined by quantification of the peak

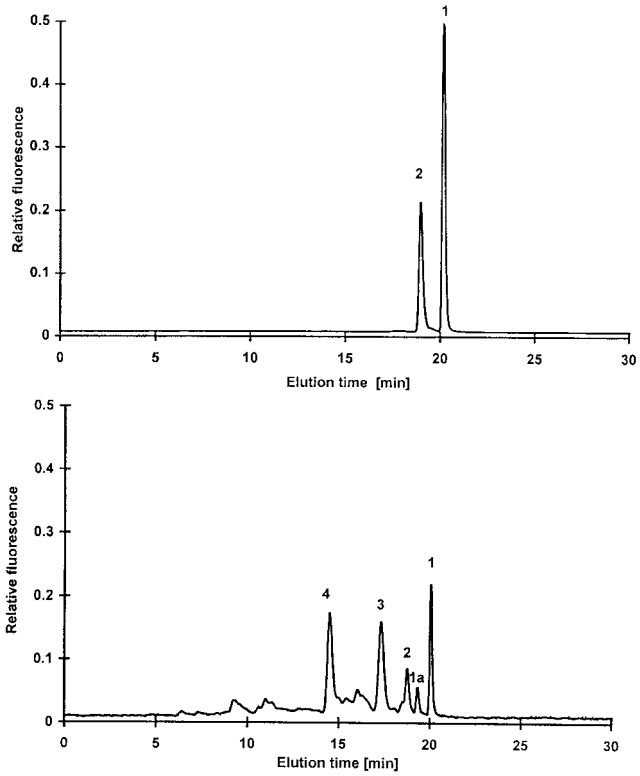

Figure 2. HPLC chromatogram of pheophytins derived from Chls of tobacco leaves. A, Wild-type tobacco; B, tobacco transformed with the *Chl P* gene in antisense orientation. Labeled peaks are: Phe a_{Phy} (1); Phe a'_{Phy} (1a); Phe b_{Phy} (1a); Phe a_{GG} (3); and Phe b_{GG} (4).

areas. As expected, the wild-type plants contained only phytylated Chl. Whereas line 21, which had wild-type-like characteristics, contained only 4% Chl_{GG}, the strongly reduced amounts of Chl of lines 10 and 6 consisted of 56% and 53% Chl_{GG}, respectively (Table I). If the phytylated portion of Chl was related to the reduced amount of total Chl in the transformants, the values for Chl_{Phy} and α-tocopherol were about equal (Table I), indicating that antisense inhibition of *Chl P* expression affects the pathways leading to Chl_{Phy} and to α-tocopherol to the same extent.

These initial data of six representative transformants reflect the variation of inhibitory effects by *Chl P* antisense gene expression in transgenic tobacco plants. The percentage of Chl_{GG} is apparently a suitable indicator for the extent of CHL P inhibition and was used to substantiate the primary examinations of the antisense inhibition upon different physiological conditions. We grew plants at a constant temperature of 22°C in light intensities of 70 and 500 μM m^{-2} s^{-1} and designated the conditions as LL and HL growth, respectively. All plants exhibited the distinct properties of either LL- and HL-exposed plants apart from their characteristic transgenic phenotype. The LL-grown plants displayed smaller and paler leaves as well as extended internodia. HL-grown plants looked more vigorous and robust and contained more spacious leaves. To test a possible developmental regulation, we analyzed leaves 3 to 10 (counting from the top).

Wild-type leaves contained exclusively Chl_{Phy}. In contrast, Chl_{GG} was detected in all transformants under conditions of irradiation and leaf development (Table II). In leaves 1 to 3, Chl_{GG} was not detectable in any transformant. The percentage of Chl_{GG} increased generally from leaves 5 to 7 (Table II) and did not increase further in leaves 8 to 13 (data not shown). Therefore, the data indicate an increasing effect of the antisense inhibition with leaf development. Furthermore, the percentage of Chl_{GG} was generally higher in transgenic plants grown under LL conditions than in the same plants cultivated under HL conditions. Under HL conditions only the three transformants, 6, 10, and 47, showed significant accumulation of Chl_{GG} in the analyzed leaves (Table II).

No significant difference was found between the portion of Chl a_{GG} and Chl b_{GG} in transformants grown under LL conditions. In HL-cultivated transgenic plants 6, 10, and 47, the degree of Chl a_{GG} was higher than that of Chl b_{GG} (Table II). As expected, the total Chl *a/b* ratio was higher in HL-grown wild-type and transgenic plants than in LL-grown plants. In LL-grown plants the Chl *a/b* ratio did not differ between the geranylgeranylated and the phytylated Chl molecules. Under HL conditions transformants contained a lower degree of Chl b_{GG} than Chl a_{GG}, resulting in a higher Chl *a/b* ratio for the geranylgeranylated molecules

Table II. *Percentage of Chl_{GG} in leaves 5 and 7 of tobacco plants containing Chl P antisense genes and control plants*

Plants were grown under HL and LL conditions, and leaf extracts were analyzed by HPLC and spectrometry. Presented are the percentages of Chl a_{GG} and Chl b_{GG} based on total Chl in the respective transgenic or control plant (Chl_{GG} + Chl_{Phy} = 100%).

Plant	HL-5		HL-7		LL-5		LL-7	
	Chl *a*	Chl *b*	Chl *a*	Chl *b*	Chl *a*	Chl *b*	Chl *a*	Chl *b*
				%				
WT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	9.4	4.1	31.6	23.0	49.9	47.7	54.9	54.1
10	0.0	0.0	12.0	7.8	39.3	36.3	57.0	57.9
20	0.5	0.5	0.7	0.7	7.2	7.0	13.0	12.7
21	0.0	0.0	1.6	2.0	2.8	2.7	9.4	9.5
24	0.0	0.0	0.0	0.0	29.9	32.3	29.1	35.0
47	9.1	5.7	11.1	7.5	25.2	22.0	22.5	29.4

(5.2 in leaf 5 of transformant 47, 6.3 in leaf 5 of transformant 6, and of 7.5 in leaf 7 of transformant 10).

Chl P Expression in CHL P-Deficient Transgenic Plants under Two Different Light Intensities

We extended our comparative biochemical and genetic analysis of transgenic plants to dependency on light intensity to substantiate the primary examinations of the effects of reduced CHL P contents in leaf 3, 5, and 7 of the transgenic plants. We chose lines 6, 10, and 20 for further analysis because they represented transgenic plants with significant macroscopic modifications. The *Chl P* RNA levels remained constant and the CHL P protein levels did not vary much during the development of leaves 3, 5, and 7 of control plants under LL and HL conditions. In the transformants the steady-state *Chl P* transcript and CHL P protein levels progressively decreased with age (Fig. 3). Steady-state *Chl P* transcript levels were generally lower in LL-grown transformants than in those grown under HL conditions and were more rapidly diminished during leaf development. The CHL P protein levels correlated with RNA content during leaf development under identical conditions. The more rapidly descending levels of the CHL P protein under LL conditions might reflect its lower stability because of fewer reduction equivalents and less substrate in these transformants.

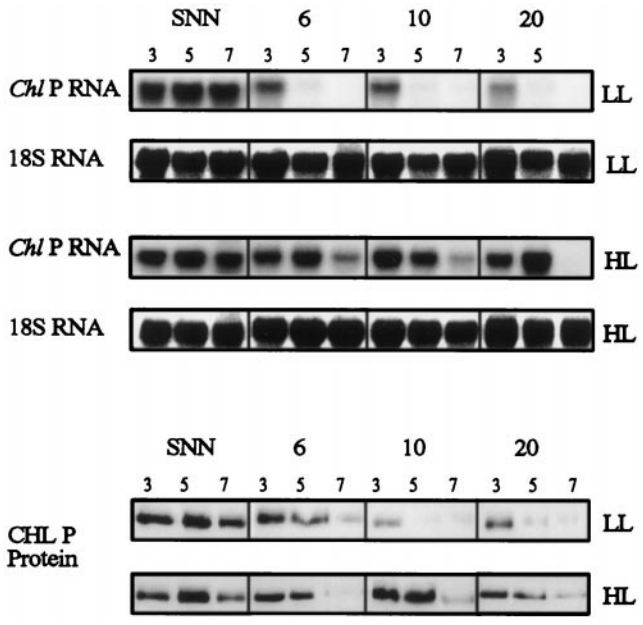

Figure 3. Expression of CHL P in transgenic (6, 10, and 20) and wild-type tobacco plants (SNN) was determined by northern and western analysis of HL- and LL-grown plants. Total RNA was isolated from leaves 3, 5, and 7 (counted from the top of each plant). Ten micrograms of RNA was loaded per lane, separated on a 1% formaldehyde-agarose gel, and hybridized to a tobacco *Chl P* cDNA probe. A cDNA probe for 18*S* rRNA was subsequently hybridized to the RNA on the same filter. Equal amounts of protein extracted from leaf 3, 5, and 7 were loaded on a SDS-polyacrylamide gel. After transfer to a nitrocellulose filter, immunodetection was performed with antiserum raised against recombinant CHL P.

Chl, Tocopherol, and Carotenoid Contents Are Developmentally Controlled in Transgenic Plants under LL and HL Conditions

Chl accumulation during leaf development was determined in leaves 3 to 10 of HL- and LL-grown transgenic and control plants (Fig. 4). Since there was no significant difference between Chl *a* and *b* under most physiological conditions (see Table II), we continued to determine only total Chl. Eight-week-old control plants reached the maximum Chl level upon LL exposure in leaf 5 (1.36 nmol/g fresh weight) and under HL exposure in the same leaf (1.85 nmol/g fresh weight). The transient increase of total Chl contents up to leaf 5 and its subsequent decrease in the transgenic lines followed the wild-type pattern. Under LL conditions the Chl content was lower only in analyzed leaves of transgenic lines 6 and 10 (maximal 30% and 40% Chl, respectively, less than the wild-type leaves). All HL-grown transformants contained significantly reduced Chl contents compared with wild-type values, particularly if older leaves were compared (e.g. 70%, 48%, and 26% less Chl in leaf 10 of transformants 6, 10, and 20, respectively, than in wild-type plants; Fig. 4).

Tocopherol content was analyzed in HL- and LL-grown control and transgenic plants (Fig. 4). Evaluation of the tocopherol content in premature leaves of control plant and of lines 20, 10, and 6 revealed that under HL conditions the plants contained 110%, 83%, 139%, and 47%, respectively, more tocopherol than under LL growth conditions. In wild-type plants the tocopherol content increased maximally up to leaf 7 under both light intensities, remained constant during plant development, and decreased progressively during senescence (data not shown). Tocopherol content was progressively reduced from the young to the older leaves in lines 6 and 10 and only slightly lower in line 20 after a similar profile during leaf development, compared with wild-type plants. The degree of lowered tocopherol levels was different in each transformant, but the relative decrease in tocopherol contents was very similar in each respective line under conditions of either LL or HL growth (Fig. 4). In leaf 10 of lines 6 and 10, tocopherol yielded approximately 20% of the wild-type contents under both growth conditions and in line 20 the yield was approximately 75%.

Comparison of the decline in Chl and tocopherol in the transgenic relative to the wild-type plants during leaf development revealed that the relative tocopherol contents were diminished to a similar extent under both light intensities and that the relative amount of total Chl was more intensively reduced with age in HL-grown than in LL-grown plants of the same transgenic lines.

The total carotenoid contents during leaf development of LL-and HL-grown transformants paralleled approximately the total Chl content. Accumulation of the carotenoid species neoxanthin, violaxanthin, lutein, and β-carotene is depicted in Table III. The amounts of zeaxanthin and antheraxanthin could not be determined by our HPLC program because Chl a_{GG} was eluted from the HPLC column simultaneously with zeaxanthin and Chl b_{GG} with antheraxanthin. All carotenoid species accumulated to lower amounts

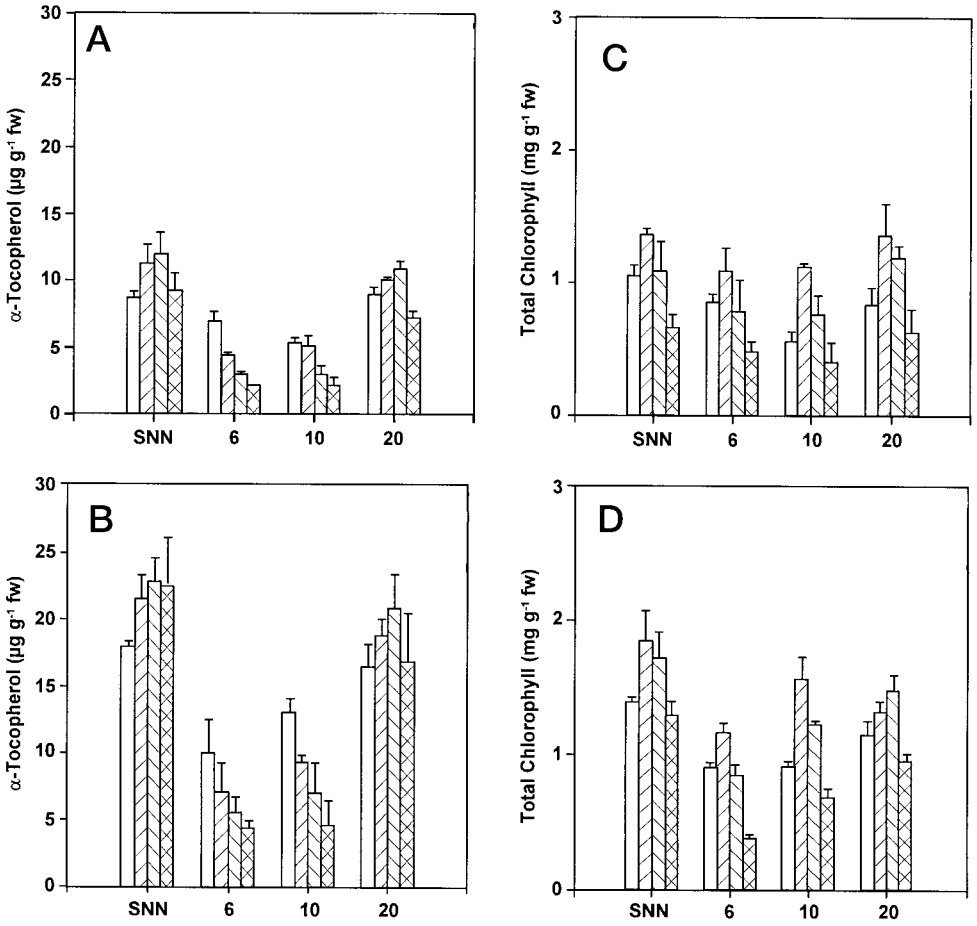

Figure 4. Contents of total Chl (C and D) and α-tocopherol (A and B) in extracts of leaf 3, 5, 7, and 10 from LL-grown (A and C) and HL-grown (B and D) transgenic tobacco plants with reduced CHL P expression (lines 6, 10, and 20) and control plants (SNN). White bars, Leaf 3; right-hatched bars, leaf 5; left-hatched bars, leaf 7; and cross-hatched bars, leaf 10. fw, Fresh weight.

in the transgenic plants grown under HL or LL conditions than in control plants. However, the reduction of lutein and β-carotene content was more pronounced in the transgenic plants relative to wild-type plants than the reduction of violaxanthin and neoxanthin. Comparing the contents of lutein and β-carotene during developmental growth of the transgenic lines, it is noticeable that both carotenoids were more diminished under HL than under LL growth conditions.

DISCUSSION

The plastidal metabolite GGPP is an intermediate in several biosynthetic pathways. It can be channeled into the Chl pathway by reduction to PhyPP and final esterification with chlorophyllide or vice versa by the initial prenylation of chlorophyllide and a subsequent reduction of Chl_{GG} (Fig. 5). GGPP can also be directed into tocopherol synthesis when PhyPP is condensed with homogentisate. CHL P catalyzes the reduction of GGPP and Chl_{GG} in vitro (Keller et al., 1998). It is not known if the enzyme accepts both substrates with the same specificity. Moreover, it remains to be seen if the same enzyme serves simultaneously the Chl- and the tocopherol-synthesizing pathways in planta.

We identified the tobacco *Chl P* cDNA sequence and introduced a binary vector harboring an antisense *Chl P* gene into tobacco to reduce specifically the enzyme activity of CHL P. This transgenic approach not only addresses the question of whether CHL P functions for two pathways but also enables a prediction on the control mechanism that distributes the enzymatic product of CHL P toward Chl or tocopherol synthesis.

The antisense inhibition of the *Chl P* expression led to an increasing proportion of Chl_{GG} in the total amount of Chl and to lower tocopherol contents during leaf development of the analyzed transformants (Table II). The deficiency of tocopherol was found synchronously with the increasing amount of Chl_{GG} (Table I). Therefore, the involvement of CHL P in the formation of Chl and tocopherol can be described as two equivalent functions. Our results obtained with the *Chl P* antisense plants are in agreement with the assumption of hydrogenation of GGPP to PhyPP and of Chl_{GG} to Chl_{Phy} and with the second function of CHL P protein in contributing to the formation of tocoph-

Table III. *Quantitative analysis of carotenoids in leaves 3, 5, 7, and 10 of control and selected transgenic lines 6, 10, and 20*

Extracts were prepared as for tocopherol determination and subjected to HPLC as described in "Materials and Methods." Compounds were identified and quantified with the help of authentic standards. Values in parentheses are SD.

Carotenoid	LL				HL			
	SNN	6	10	20	SNN	6	10	20
	μg g⁻¹ fresh wt							
Neoxanthin								
Leaf								
3	18.5 (0.64)	13.2 (1.38)	12.8 (1.55)	15.7 (0.43)	20.6 (1.03)	18.3 (1.83)	19.0 (3.99)	13.4 (1.48)
5	28.5 (0.98)	25.4 (0.20)	22.2 (0.65)	28.1 (3.81)	29.4 (1.48)	27.0 (2.10)	26.7 (1.79)	20.8 (1.87)
7	28.0 (3.83)	28.1 (0.20)	28.2 (1.24)	26.0 (0.65)	27.8 (3.23)	27.4 (2.23)	33.8 (5.17)	21.7 (2.43)
10	17.3 (1.47)	26.5 (0.59)	20.6 (3.24)	16.9 (2.03)	25.2 (1.79)	15.7 (4.72)	31.3 (8.06)	18.4 (2.85)
Violaxanthin								
Leaf								
3	28.3 (0.85)	21.0 (1.38)	20.4 (0.69)	24.9 (0.64)	33.0 (1.47)	30.7 (5.50)	32.5 (6.86)	25.5 (2.23)
5	34.6 (1.57)	29.7 (0.59)	24.7 (0.65)	35.4 (4.98)	45.9 (1.03)	41.1 (1.31)	39.6 (4.26)	38.2 (4.10)
7	27.8 (3.43)	24.0 (1.18)	22.2 (1.22)	26.6 (1.29)	38.5 (4.25)	30.1 (4.19)	35.6 (6.55)	35.2 (4.15)
10	15.9 (1.77)	17.3 (0.39)	13.5 (1.01)	15.8 (2.29)	29.4 (3.40)	13.2 (3.28)	22.4 (7.08)	21.9 (5.55)
Lutein								
Leaf								
3	89.1 (6.77)	59.8 (7.42)	59.6 (5.26)	77.4 (1.24)	124.8 (4.75)	87.9 (6.11)	109.8 (24.86)	80.7 (9.22)
5	132.3 (8.30)	103.5 (3.93)	87.9 (4.17)	129.8 (20.06)	161.0 (7.24)	105.0 (16.16)	130.1 (12.97)	114.0 (11.54)
7	121.0 (20.73)	81.0 (7.21)	78.9 (2.52)	111.4 (2.13)	142.9 (20.11)	83.0 (13.68)	129.3 (26.90)	113.1 (13.80)
10	75.6 (5.40)	62.4 (3.06)	47.8 (7.43)	64.7 (7.43)	127.7 (9.84)	39.2 (7.42)	89.7 (29.04)	87.1 (9.10)
β-Carotene								
Leaf								
3	52.7 (2.93)	38.5 (3.16)	32.5 (2.08)	49.0 (9.03)	76.7 (2.06)	48.0 (4.21)	60.3 (12.51)	51.2 (4.79)
5	69.2 (2.53)	51.9 (2.37)	46.5 (3.03)	71.8 (11.10)	93.7 (4.03)	53.3 (9.68)	68.7 (5.96)	70.0 (6.38)
7	62.4 (9.45)	32.8 (4.74)	31.5 (2.41)	59.1 (2.10)	83.9 (11.15)	35.8 (8.21)	59.1 (14.33)	66.2 (7.08)
10	36.8 (3.24)	18.3 (1.89)	15.7 (3.24)	31.4 (3.23)	72.8 (6.34)	11.9 (2.63)	30.5 (12.79)	46.1 (5.85)

erol. The recombinant Arabidopsis CHL P protein expressed in *E. coli* was shown to be active in both the reduction of GGPP and of Chl_{GG} (Keller et al., 1998). The *R. capsulatus bchP* insertion mutant fails in the hydrogenation step from geranylgeraniol to phytol, the esterifying alcohol of bacteriochlorophyll (Bollivar et al., 1994).

Under increased light intensities, wild-type plants and the analyzed transformants accumulated more Chl (between approximately 35% in leaves of wild-type plants and 12% in those of line 6) and up to 2 times more tocopherol (Fig. 4). Since the wild-type contents of CHL P seemed to be similar under LL and HL conditions in leaves of the same age, it is suggested that CHL P expression does not normally limit the supply of precursor for Chl and tocopherol during plant development. However, as was apparent from the analysis of the transgenic lines, significant reduction of the amount of CHL P by antisense RNA synthesis affects the levels of both biomolecules in a light and developmentally dependent manner. It is remarkable that transformants with diminished amounts of CHL P not only contained reduced contents of phytylated Chl but also contained less total Chl than wild-type plants. It is assumed either that Chl_{GG} is not associated as stably as Chl_{Phy} in the photosynthetic pigment-binding proteins, re-

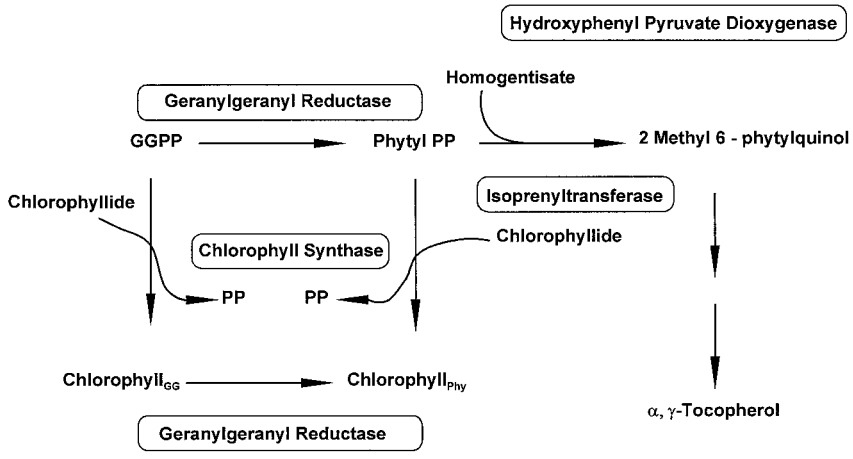

Figure 5. Scheme of the branched pathway starting from GGPP to α-tocopherol, γ-tocopherol, or Chl_{Phy}. CHL P, Chl synthase, and an isoprenyl transferase are indicated. CHL P uses GGPP and Chl_{GG} as substrates and directs PhyPP to the tocopherol- and the Chl-synthesizing pathway.

sulting in a faster Chl breakdown (analogous to the assumption of labile bacteriochlorophyll$_{GG}$-protein complexes; Bollivar et al., 1994), or that the reduced synthesis of isoprenoid and Chl precursors is indirectly caused by the antisense inhibition of CHL P expression.

The percentage of phytylated Chl was generally higher in HL transformants than in the same LL-grown transgenic lines, which could be explained by several factors. First, if Chl synthase activity were higher under HL than under LL conditions, more PhyPP but also more accumulating GGPP could be channeled into the Chl-synthesizing pathway. Chl$_{GG}$ competes with GGPP for the residual hydrogenation activity of CHL P, leading to more Chl$_{Phy}$. Second, an increasing pool of NADPH or other reducing biomolecules due to the stimulated photosynthesis in HL-exposed leaves could activate CHL P, which should lead to more Chl$_{Phy}$ and α-tocopherol. However, it seems more likely that the low Chl$_{GG}$ levels in HL-grown transgenic plants result from photooxidation of Chl$_{GG}$ so that Chl$_{Phy}$ remains preferentially. It is unknown whether HL conditions cause destabilization of pigments, especially of Chl$_{GG}$ in the *Chl P* antisense plants.

Chl *a* and *b* were phytylated to the same extent in most transformants grown under LL conditions. Some transformants exposed to HL conditions showed more Chl a_{GG} than Chl b_{GG}, which is indicative for a preferential supply of Chl for the reaction center core complex under increasing light intensities. This would mean that Chl *a* exhibits a faster turnover than Chl *b* in HL-exposed transformants, most likely in the reaction center of the photosystems and to lesser extent in the antenna complexes.

Approximately 2 times more tocopherol accumulated upon increased light intensity in wild-type and transgenic plants compared with those exposed to lower light intensities. It has been shown previously that HL exposure modulates the tocopherol content in photosynthetic membranes (Lichtenthaler, 1979). The antioxidant function of tocopherol is expected to scavenge photogenerated singlet oxygen or other organic radicals in photosynthetic membranes. Exposure to enhanced light intensities increases the risks of photodynamic damage. An increased requirement of tocopherol under HL conditions is most likely adjusted by stimulation of its synthesis in response to light intensities. The analysis of the transgenic lines with reduced CHL P expression did not provide evidence that HL exposure would lead to preferential channelling of PhyPP under limiting synthesis of this precursor into the tocopherol-synthesizing pathway. However, the consequences of insufficient levels of tocopherol became apparent in the transgenic plants with reduced CHL P content. The transformants grew slower and showed a bleached phenotype.

Lower CHL P activity could result in accumulation of GGPP, which can also be directed into carotenoid synthesis. However, the analyzed carotenoid levels were lower in the transformants than in the wild-type plants and almost paralleled the reduction in Chl content. The assembly of Chl$_{GG}$ instead of Chl$_{Phy}$ with the pigment-binding proteins does not significantly modify the composition of carotenoids in the pigment-protein complexes. Our observation reflects the synchronized need for both pigment fractions and the pigment-binding proteins to stabilize the photosynthetic complexes and is consistent with observations of the parallel loss of pigments and pigment-binding proteins in mutants with deficiencies in Chl or carotenoid synthesis (Plumley and Schmidt, 1995).

In conclusion, we demonstrate here, for the first time to our knowledge, that deficiency of CHL P simultaneously affects two pathways, leading to a decline in tocopherol and phytylated Chl contents and in the level of total Chl molecules. Lack of tocopherol could destabilize the thylakoid membrane and negatively influence the photosynthetic machinery. Analysis of the photoprotective functions of tocopherol and other antioxidants under various environmental conditions and for photosynthesis in the *Chl P* antisense plants is currently being performed. Transgenic plants with reduced tocopherol contents seem to be an appropriate model with which to investigate how the transformants prevent the deleterious photo effects and if and how they compensate for the tocopherol deficit.

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