Plant Physiol. (1997) 113: 1113–1124

Restriction of Chlorophyll Synthesis Due to Expression of Glutamate 1-Semialdehyde Aminotransferase Antisense RNA Does Not Reduce the Light-Harvesting Antenna Size in Tobacco[1]

Heiko Härtel*, Elisabeth Kruse, and Bernhard Grimm

Institut für Biologie, Lehrstuhl für Pflanzenphysiologie, Humboldt-Universität zu Berlin, Unter den Linden 6, Sitz: Philippstrasse 13, D-10099 Berlin, Germany (H.H.); and Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, D-06466 Gatersleben, Germany (E.K., B.G.)

The formation of 5-aminolevulinate is a key regulatory step in tetrapyrrole biosynthesis. In higher plants, glutamate 1-semialdehyde aminotransferase (GSA-AT) catalyzes the last step in the sequential conversion of glutamate to 5-aminolevulinate. Antisense RNA synthesis for GSA-AT leads to reduced GSA-AT protein levels in tobacco (*Nicotiana tabacum* L.) plants. We have used these transgenic plants for studying the significance of chlorophyll (Chl) availability for assembly of the light-harvesting apparatus. To avoid interfering photoinhibitory stress, plants were cultivated under a low photon flux density of 70 μmol photons m^{-2} s^{-1}. Decreased GSA-AT expression does not seem to suppress other enzymic steps in the Chl pathway, indicating that reduced Chl content in transgenic plants (down to 12% of the wild-type level) is a consequence of reduced GSA-AT activity. Chl deficiency correlated with a drastic reduction in the number of photosystem I and photosystem II reaction centers and their surrounding antenna on a leaf area basis. Different lines of evidence from the transgenic plants indicate that complete assembly of light-harvesting pigment-protein complexes is given preference over synthesis of new reaction center/core complexes, resulting in fully assembled photosynthetic units with no reduction in antenna size. Photosynthetic oxygen evolution rates and in vivo Chl fluorescence showed that GSA-AT antisense plants are photochemically competent. Thus, we suggest that under the growth conditions chosen during this study, plants tend to maintain their light-harvesting antenna size even under limited Chl supply.

In higher plants light energy is harvested by specific pigment-protein complexes that efficiently transfer excitation energy to reaction centers of PSII and PSI, where charge separation takes place. Two major groups of pigment proteins are distinguished: (a) the Chl *a*-binding chloroplast-encoded apoproteins of the reaction center/core complexes comprising the polypeptides P700A, P700B, D1, D2, CP43, and CP47 (Mullet, 1988); and (b) the Chl *a*- and Chl *b*-binding apoproteins of LHCs, which are encoded by nuclear genes of the *Lhc* family (Jansson, 1994). According to the gene-related nomenclature proposed by Jansson et al. (1992), the polypeptides Lhcb1 to Lhcb6 are associated with PSII, and the polypeptides Lhca1 to Lhca4 are associated with PSI. The most prominent antenna complex is LHCII, the major peripheral LHC of PSII, probably consisting of mixed trimers of the polypeptides Lhcb1 and Lhcb2 (Jansson, 1994). In mature thylakoids LHCII binds approximately 50% of total Chl and most of Chl *b*. The minor (probably monomeric) polypeptides Lhcb3 to Lhcb6, which are assumed to be directly attached to the PSII core complex, presumably form the inner LHCII antenna.

Chl is synthesized by a multienzymic pathway that branches at the tetrapyrrole precursor protoporphyrin IX to either Chl or heme. Light affects Chl biosynthesis at steps involving both ALA and chlorophyllide formation (for reviews, see Beale and Weinstein, 1990; von Wettstein et al., 1995). Chl seems to be necessary for stabilizing apoproteins of both the reaction center/core complex (Klein et al., 1988; Herrin et al., 1992; Kim et al., 1994) and the LHCs (Argyroundi-Akoyunoglou et al., 1982; Mathis and Burkey, 1989; Herrin et al., 1992). A coordinated synthesis of Chls and Chl-binding proteins is suggested. Only a small amount of LHCII accumulates in a Chl *b*-deficient mutant, but its LHC transcript levels resemble that of wild type (Apel and Kloppstech, 1980; Mathis and Burkey, 1989). On the other hand, despite drastically reduced levels of mRNA encoding LHCII (down to 5% of the level of wild-type tobacco [*Nicotiana tabacum* L.]) due to expression of its antisense RNA, LHCII accumulated in comparable amounts in the primary transformants compared with the wild type (Flachmann and Kühlbrandt, 1995). This supports the hypothesis that LHCII content is not primarily governed by transcript synthesis, but by posttranscriptional stabilization, for which Chl is thought to be essential. However, the mechanism by which Chl is assembled with

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant nos. Ho 1757/1 and Gr 936/3+4).

* Corresponding author; e-mail heiko=haertel@rz.hu-berlin.de; fax 49–30–2093–6337.

Abbreviations: ALA, 5-aminolevulinate; ALA-D, 5-aminolevulinate dehydratase; Chl, chlorophyll; CH42, CHL I subunit of Mg chelatase; GLU-TR, glutamyl tRNA reductase; GSA-AT, glutamate 1-semial-dehyde aminotransferase; HMBS, 1-hydroxymethylbilane synthase; LHC, light-harvesting pigment-binding complexes; LHCI and LHCII, light-harvesting pigment-binding complexes of PSI and PSII, respectively; F_v and F_m, variable and maximal fluorescence yield, respectively, in the dark-adapted state.

pigment-protein complexes of the photosynthetic apparatus is not understood.

In addition to Chls, carotenoids are constituents of pigment-protein complexes. β-Carotene is mainly an integral part of the reaction center/core complex, whereas xanthophylls, which represent the majority of leaf carotenoids, are located mainly within LHCs. Carotenoids are important for LHC structure and stability (Plumley and Schmidt, 1987; Paulsen et al., 1990; Kühlbrandt et al., 1994) and have a light-harvesting as well as a photoprotective function. Although xanthophylls and Chls are arranged within the same protein complexes, an understanding of the simultaneous regulation of pigment and apoprotein synthesis remains elusive. Recently, Plumley and Schmidt (1995) hypothesized that synthesis of both Chls and at least some xanthophylls may occur directly within hydrophobic domains of light-harvesting pigment proteins to ensure balanced synthesis of pigments and pigment proteins.

The roles of Chl in regulating formation of pigment-protein complexes have largely been elucidated from kinetic studies of greening plants, plants grown under intermittent long-term-dark/short-term-light regimes, or mutant plants that are defective in certain steps of the Chl biosynthetic pathway. In this paper we investigated Chl-deficient transgenic plants that synthesize antisense RNA for GSA-AT, one of the early enzymes for Chl synthesis. The formation of ALA from glutamate is considered to be rate-limiting in the biosynthesis of tetrapyrroles (for reviews, see Kannangara et al., 1994; von Wettstein et al., 1995). GSA-AT catalyzes the conversion of glutamate 1-semialdehyde to ALA (Grimm, 1990). Expression of GSA-AT antisense RNA in tobacco plants results in a varying decrease of GSA-AT activity, and a subsequent decline in Chl content is usually observed (Höfgen et al., 1994). An advantage of the GSA-AT antisense plants is the genetically defined partial decrease in ALA synthesis and the resulting deprivation of the overall capacity of Chl formation. Therefore, these antisense plants provide an excellent model system in which to analyze modulated accumulation of pigment proteins relative to the Chl content. In the present study we show that reduced Chl levels in response to a partial suppression of GSA-AT expression correlate with a reduction in the number of reaction centers and their surrounding light-harvesting antenna per unit of leaf area. It is shown that Chl deficiency in transgenic plants does not prevent complete assembly of both photosystems, although they are reduced in amount.

MATERIALS AND METHODS

Wild-type tobacco (*Nicotiana tabacum* L. cv SNN) plants and primary transformants expressing GSA-AT antisense RNA that were derived from sterile shoot cultures were used for experiments. The plants were cultivated in soil with a 14-h/10-h light-dark cycle at 25°C and 65% RH in a growth chamber. During the period of growth, plants were exposed to a photon flux density of 70 μmol photons m^{-2} s^{-1}. If not stated otherwise, the 6th to 10th leaves of 6- to 8-week-old plants were used for measurements. Wild-type plants were usually in the 15- to 17-leaf stage, transformant no. 42 in the 12- to 14-leaf stage, and the other transformants between these two developmental stages. The construction of the GSA-AT antisense gene transformants was described by Höfgen et al. (1994).

RNA Analysis

Total RNA was isolated essentially as described previously (Chomczinsky and Sacchi, 1987). Aliquots of 15 μg of RNA were used for northern-blot analysis. cDNA sequences used encode GSA-AT (Höfgen et al., 1994), GLU-TR (Bougri and Grimm, 1996), ALA-D (O. Bougri and B. Grimm, unpublished data), and CH42 (J. Papenbrock, H.-P. Mock, and B. Grimm, unpublished data). cDNA fragments were radioactively labeled with [^{32}P]dCTP by random priming.

Native Green-Gel Electrophoresis

Thylakoids were isolated essentially as described by Camm and Green (1982). Membranes were solubilized with 0.45% octyl glucoside, 0.45% decyl maltoside, and 0.1% lithium dodecyl sulfate in 2 mM Tris maleate, pH 7.0, 10% glycerol at a Chl concentration of 0.45 mg mL^{-1} for 30 min on ice (Allen and Staehelin, 1991). Solubilized pigment-protein complexes representing 8 μg of total Chl were separated by mildly denaturing PAGE (Andersson et al., 1982). Electrophoresis was performed at 4°C with 7.5 mA per gel for 3 h until the free pigments had migrated about two-thirds of the gel length. Three independent thylakoid preparations from tobacco wild type and the GSA-AT transformants were analyzed in different gels, and the lanes were scanned at 675 nm with a UV/visible spectrophotometer equipped with a gel scanner (Specord M500, Zeiss).

Pigment Assay and Protein Analysis

Leaf discs were punched with a cork borer and rapidly frozen in liquid nitrogen for subsequent pigment and protein determination. Pigments were analyzed by HPLC as described previously (Härtel et al., 1996a). Total leaf protein was extracted as described by Kruse et al. (1995). Western-blot analysis was performed as described by Härtel et al. (1996a), except that total leaf extracts were used.

Estimation of the Number of PSI and PSII Reaction Centers

Leaf material was ground in ice-cold buffer (pH 7.5) containing 50 mM Tricine, 5 mM $MgCl_2$, 10 mM NaCl, 400 mM sorbitol, and 0.2% BSA. The homogenate was rapidly filtered through one layer of nylon sheet and centrifuged at 10,000*g* for 2 min. This procedure ensures pelleting of fully developed thylakoids as well as etioplast membranes. The pellet was washed once in buffer lacking sorbitol and resuspended in isolation buffer. The Chl content of the extracts was determined in 80% acetone according to the method of Lichtenthaler (1987). The P700 content of Triton X-100 (1%, w/v)-treated thylakoids was determined from the ferricyanide-oxidized minus the sodium ascorbate-

reduced difference in A_{702} (Marsho and Kok, 1980) using a dual-beam spectrophotometer (ZWSII, Sigma Eppendorf, Hamburg, Germany). For calculation a molar extinction coefficient of 64 mM^{-1} cm^{-1} was used (Hiyama and Ke, 1972).

The number of PSII reaction centers was estimated from the atrazine binding sites of PSII by a procedure similar to that described by Tischer and Strotmann (1977). [^{14}C]-Atrazine was added at various concentrations to 1-mL samples. The Chl concentration in each sample was 50 μg mL^{-1}. For equilibration, samples were incubated in dim light for 30 min at room temperature and then centrifuged at 15,000g with an Eppendorf microfuge for 3 min. The total amount of atrazine bound to the thylakoids was determined by measuring the content of [^{14}C]atrazine in the supernatant and, to improve accuracy, also in the pellet using a liquid scintillation counter (LS 6000 SC, Beckman). The number of binding sites was determined from double-reciprocal plots of free [^{14}C]atrazine versus bound [^{14}C]atrazine (nmol mg^{-1} Chl).

Chl Fluorescence Measurements

Low-temperature (77 K) Chl fluorescence emission spectra of thylakoid suspensions were recorded using a spectrophotometer (F-4500, Hitachi, Tokyo, Japan). After 10 min of darkening, samples adjusted to 2.5 μg Chl mL^{-1} were rapidly frozen in liquid nitrogen. Excitation and emission slit widths were 5 and 2.5 nm, respectively. Room-temperature Chl fluorescence was monitored from attached leaves as described by Härtel et al. (1993).

O_2 Evolution

Rates of O_2 evolution of leaf segments were measured polarographically at 25°C with a leaf-disc O_2 electrode (Bachofer, Reutlingen, Germany) in saturating CO_2 (5%, v/v). Leaf discs of 9 cm^2 were used for measurements to ensure coverage of a representative leaf section. O_2 evolution of thylakoids suspended in a buffer solution of 50 mM Tricine, 330 mM sorbitol, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 20 mM Fe-EDTA, and 20 mM NaCl (pH 7.5) were measured using a Clark-type electrode. Uncoupled whole-chain (PSI + PSII) electron transport activity was determined in the presence of 2 mM $K_3Fe(CN)_6$ and 3 mM methylamine. Light was provided by a cold light source (KL 1500, Schott, Mainz, Germany). PAR was measured as incident photon flux density with a quantum sensor (LI-189A, Li-Cor, Lincoln, NE).

RESULTS

Growth of GSA-AT Antisense Plants

A cDNA sequence encoding GSA-AT was expressed in tobacco in reverse orientation under control of the cauliflower mosaic virus 35S promoter as described by Höfgen et al. (1994). As shown in Figure 1, transgenic plants expressing GSA-AT antisense RNA showed great variability in Chl content. Depending on the extent of chlorosis, antisense GSA-AT transformants exhibited a more or less retarded growth. Transgenic lines chosen for our studies have been investigated previously (Höfgen et al., 1994) and covered a broad range of decreasing Chl content. Furthermore, they kept a stable phenotype for several generations and years. Transformed plant phenotypes ranged from uniformly pale yellow (no. 42) or patchy pale leaves (no. 25) to plants with white tissue along the leaf veins (no. 57), whereas leaf shape remained similar to that of wild type. Although antisense gene expression is under control of the cauliflower mosaic virus 35S promoter, the difference in Chl deficiency in leaf cells of the transformants nos. 25, 56, and 57 might reflect cell diversity in age, function, and Chl synthesis. However, the progeny of their T_1, T_2, and T_3 generations contained the same pattern of leaf variegation and pigment intensity.

Figure 1. Phenotypes of wild-type tobacco (SNN) and its GSA-AT antisense transformants after a 6-week period of cultivation in a growth chamber (10 h of dark/14 h of light at 70 μmol photons m^{-2} s^{-1}, 25°C). Upper row from left to right, SNN, transformants nos. 14 and 40; lower row from left to right, transformants nos. 25, 57, 42, and 56.

Only primary transformants were used for our studies. All plants regenerated from leaves of primary transformants retained their original phenotypes. A few transformants (out of more than 100 different primary transformants) did not tolerate greenhouse conditions and died before flowering. Therefore, to maintain stable phenotypes and to avoid possibly interfering photoinhibitory effects, plants were cultivated under low light (70 μmol photons m^{-2} s^{-1} PAR).

GSA-AT Antisense RNA Synthesis Results in a Variable Reduced Chl Content and a Simultaneously Reduced Carotenoid Synthesis

Chls and carotenoids extracted from leaves represent the average pigment content in the whole leaf tissue. The Chl content (determined on a leaf area basis) in mature leaves of transgenic plants was reduced between 88 (no. 42) and 9% (no. 58) compared with the wild type (Fig. 2A). The Chl *a*/*b* ratio was remarkably constant even throughout an increase of more than 8-fold in the Chl content per unit of leaf area among the various transgenic plants. The Chl *a*/*b* ratio of the outer (pale) and inner (green) leaf section in trans-

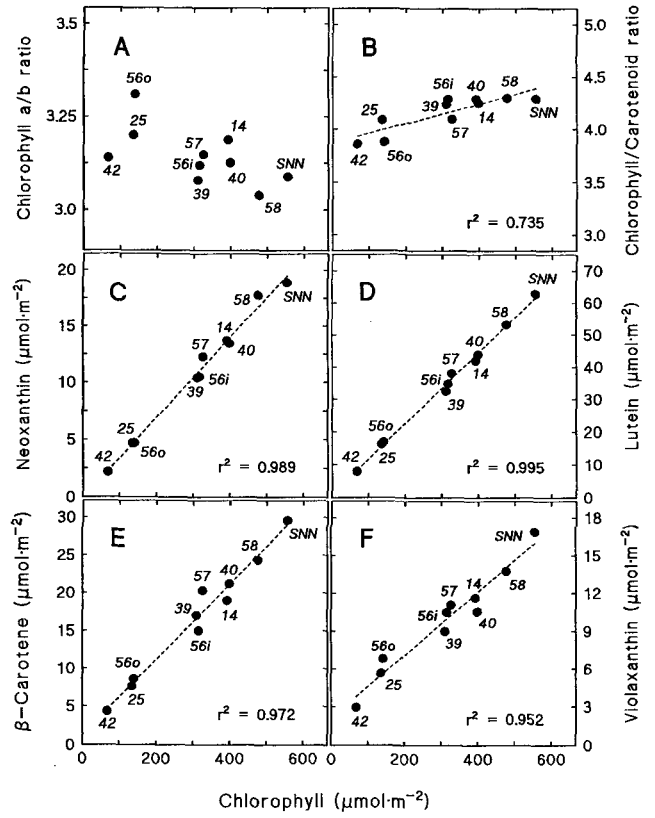

Figure 2. Chl *a/b* ratio (A), Chl/carotenoid ratio (B), and neoxanthin (C), lutein (D), β-carotene (E), and violaxanthin (F) contents as a function of Chl content determined in leaves of wild-type tobacco (SNN) and various GSA-AT transformants. Numbers at the data points refer to different transformants, except for the transformant no. 56, for which 56i and 56o stand for the inner dark-green and the outer yellow-green leaf tissues, respectively. The data are mean values (SD ≤ 12%) obtained with two sets of plants. In each set three plants were individually analyzed. The dashed lines correspond to the first-order regression to the data.

formant no. 56 was also not distinctly different, although the Chl content per leaf area showed drastic differences between both sections (Fig. 2A; see also Fig. 1). A similar situation is also predicted to occur for the pale and green areas of transformants nos. 57 and 25. A minor increase in the Chl/carotenoid ratio with Chl content was observed from a minimum in no. 42 (3.89) to a maximum in wild type (4.28; Fig. 2B). Detailed analysis of individual carotenoids revealed a highly linear correlation between the contents of Chl and neoxanthin (r^2 = 0.989; Fig. 2C), lutein (r^2 = 0.995; Fig. 2D), and β-carotene (r^2 = 0.972; Fig. 2E). The regression lines cross the *y* axis close to the origin, implicating a strict coordination in synthesis and turnover of these carotenoids and Chls in mature leaves. The amount of violaxanthin (Fig. 2F) indicated a somewhat less stringent relationship of this fraction of carotenoids on Chl content, with minor increases in the violaxanthin/Chl ratio in most chlorotic transformants. Under the growth conditions neither antheraxanthin nor zeaxanthin accumulated in any of the plants.

To investigate the dynamic interaction between Chl and carotenoid synthesis and stability, pigments were measured as a function of leaf age (from leaf 2 to the fully expanded, mature leaf 10). Maximal levels of Chl were found in leaves 6 and 8 of both wild-type plants and in the most chlorotic transformant, no. 42 (Table I). A transition in the Chl *a/b* ratio from 3.7 (leaf 2) to 3.1 (leaf 6) was measured in wild type, and a transition from 4.5 to 3.2 was measured in no. 42 (data not shown). In the wild type total carotenoids accumulated almost in parallel with Chls. The ratios of Chls to carotenoids are 3.6 and 4.2 in leaves 2 and 6, respectively. In contrast, considerably lower levels of Chls relative to carotenoids were present in younger leaves (2 and 4) of transformant no. 42. Herein Chl content increased only modestly with maturation. It is interesting that carotenoid levels in leaf 2 of no. 42 were comparable to those of leaf 2 of the wild type on a fresh weight basis (Table I). The carotenoid content subsequently strongly decreased relative to Chl from leaves 2 to 6, resulting in an increase in the Chl/carotenoid ratios from 1.8 (leaf 2) to 3.8 (leaf 6). Under the experimental conditions used, Chl *a/b* as well as Chl/carotenoid ratios in leaf 6 reached that of mature leaves in both wild-type and transgenic plants.

GSA-AT Antisense RNA Synthesis Does Not Suppress Expression of Other Selected Genes Involved in Chl Synthesis

For the analysis of the effect of suppressed GSA-AT expression on other Chl-synthesizing enzymes, we selected the representative transgenic lines nos. 57, 25, and 42, with severely reduced GSA-AT activities (Höfgen et al., 1994) and Chl contents (Fig. 2). Steady-state levels of mRNAs

Table I. *Chl and carotenoid contents along leaf development*

Pigments from leaves 2, 4, 6, 8, and 10 of 8-week-old plants of wild-type tobacco (SNN) and the transformant no. 42 were extracted with acetone and separated by HPLC analysis. Values represent means ± SD from four different plants.

Leaf	SNN		Transformant No. 42	
	Chls	Carotenoids	Chls	Carotenoids
	nmol g⁻¹ fresh wt			
2	449.0 ± 40.2	125.8 ± 14.8	202.6 ± 30.8	114.5 ± 25.8
4	678.0 ± 78.9	167.0 ± 26.6	235.9 ± 40.2	143.8 ± 36.2
6	1192.3 ± 301.2	275.7 ± 32.9	297.6 ± 31.2	88.4 ± 15.1
8	1174.8 ± 221.7	276.4 ± 30.9	284.7 ± 28.8	79.7 ± 10.4
10	1120.0 ± 100.4	269.4 ± 24.6	256.6 ± 23.8	69.8 ± 8.1

encoding GLU-TR, ALA-D, and CH42 were determined relative to the wild type using extracts of leaves 2, 4, and 6 (from the top of each plant). Young leaves were preferentially chosen for analysis of specific RNAs and proteins since Chl synthesis is most active during early stages of leaf development (He et al., 1994). GLU-TR and ALA-D are enzymes in close proximity to GSA-AT in the biosynthetic pathway. Gene expression or protein stability of these enzymes could be affected by a reduction in GSA-AT content. Control of ALA and Chl synthesis could occur at the levels of GLU-TR and Mg chelatase, respectively.

RNA was extracted from three different sets of plants and analyzed by northern-blot hybridization. Figure 3 shows a representative result. GSA-AT mRNA content decreased in older wild-type leaves, indicating a developmental control of transcript abundance. GSA-AT mRNA levels were lower in leaf tissues of all transformants and in general followed the developmentally dependent wild-type pattern. The GSA-AT transcript levels in no. 42 were below the detection limit. An additional slower-migrating band appeared in no. 57, which presumably represents the longer antisense transcript. The content of GSA-AT mRNA significantly correlates with the phenotype of transformants analyzed.

Consistent with previous observations in higher plants (He et al., 1994), maximal abundance of other RNAs encoding Chl-synthesizing enzymes was observed in younger leaves and dropped to minimal levels in older leaves of the wild type (Fig. 3). The transcript levels for GLU-TR, ALA-D, and CH42 followed comparable patterns in wild-type and transgenic plants. The differences in the abundance of these mRNA species between transformants and the wild type were marginal.

To explore the correlation of modified GSA-AT content with protein levels of other enzymes involved in Chl biosynthesis, wild type and transformants were examined by western-blot analysis. Equal amounts of leaf protein were separated by SDS-PAGE, blotted, and subsequently quantified with polyclonal monospecific antisera against GSA-AT, ALA-D, HMBS, or CH42. An antiserum against plant GLU-TR is not available at present. Wild-type leaves 2 to 6 possessed roughly constant levels of GSA-AT proteins (Fig. 4). Reduced amounts of GSA-AT were observed in premature leaves of transformants and were further diminished in older leaves. The higher GSA-AT protein level in no. 42 relative to its very low mRNA level (compare Fig. 3) might be explained by increasing protein stability. The lower contents of GSA-AT were directly proportional to the degree of bleached phenotypes of transformants. Contents of other enzymes of the Chl pathway were either similar (ALA-D and CH42) or even higher (HMBS) in transformants than in the wild type. Accumulation of Chl precursors (between ALA and Mg-protoporphyrin monomethylester) was not detected in transformants (data not shown). Therefore, expression and activity of enzymes for Chl synthesis other than GSA-AT apparently are adequate and do not account for reduced Chl contents in GSA-AT antisense plants. We conclude that reduced pigmentation and retarded growth of the transgenic plants directly correlates with inhibition of GSA-AT expression by antisense RNA, resulting in deficient ALA synthesis.

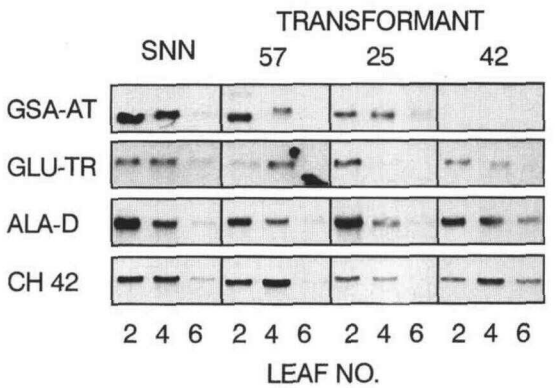

Figure 3. Expression of genes involved in Chl biosynthesis. For northern-blot analysis of mRNA encoding various enzymes involved in Chl synthesis, total RNA was extracted from leaves 2, 4, and 6 of wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42. RNA was hybridized with cDNA encoding GSA-AT, GLU-TR, ALA-D, and CH42.

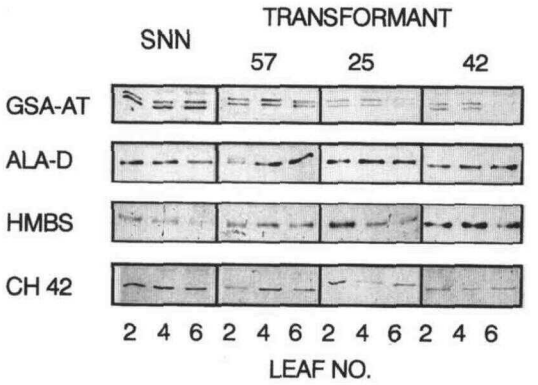

Figure 4. Protein levels of enzymes involved in Chl biosynthesis. Total leaf protein was extracted from leaves 2, 4, and 6 of wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42, and analyzed by western blotting. Polyclonal antibodies against GSA-AT, ALA-D, HMBS, and CH42 were used. In each lane an equal amount of leaf tissue (10 μg of protein) was loaded.

GSA-AT Antisense RNA Affects Expression of Genes for Chl-Binding Proteins

Heme and Chl are the final products of tetrapyrrole biosynthesis, but 100 times more Chl is formed relative to heme (Falbel and Staehelin, 1994; Kruse et al., 1995). Thus, impaired tetrapyrrole biosynthesis should visibly result in reduced Chl contents. Chl is accepted to be important for the stabilization of pigment proteins of reaction centers and LHCs. Therefore, we determined the expression of Chl-binding proteins during leaf development. Equal amounts of protein extracted from the wild type and antisense transformants were analyzed with polyclonal antibodies for contents of the PSII reaction center protein D1 and for major LHCII apoproteins. The accumulation pattern for the minor Lhcb4 and Lhca2 apoproteins was explored with the monoclonal CMp antibody from the Carlsberg laboratory

(Knoetzel and Simpson, 1991), which cross-reacts with both proteins. As shown in Figure 5, the amount of D1 and of major LHC subunits increased with leaf age in all plants analyzed. In transformant no. 42, D1 accumulated to only 10 to 15% of the wild-type level, and in no. 25 it accumulated to about 30 to 35%. Compared with control plants, the fractional contents of the major LHCII apoproteins and the minor proteins Lhcb4 and Lhca2 were simultaneously reduced in these transgenic lines on a total protein basis.

To gain further information on the mechanism that controls the assembly of the pigment-protein complexes, steady-state levels of *Lhc* mRNA from leaves of increasing maturation (leaves 2, 4, 6, and 8) of the same plants were analyzed (Fig. 6). *Lhc* transcripts showed developmentally controlled transient increases in leaves 4 and 6. In transformants nos. 25 and 42, the levels of *Lhc* mRNA were significantly enhanced relative to that of wild type. The steady-state levels of these mRNA species in leaf 6 of wild type and transformant no. 42 differed by at least a factor of 5, indicating marked changes in transcriptional activity of *Lhc* genes or the stability of their transcripts in chlorotic GSA-AT antisense plants. As is evident from Figure 6, the *psbA* RNA levels of the wild type and the transformants nos. 57 and 25 increased with leaf maturation. In comparison with the wild type the mRNA content for the D1 protein was elevated in premature leaves of no. 42. Although the mechanistic basis for the elevated RNA levels of pigment proteins in Chl-deficient GSA-AT transformants remains elusive, the elevated levels show that these mRNAs are not a limiting factor for pigment-protein synthesis.

In a next set of experiments we determined the abundance of antenna and reaction center proteins in mature leaves of GSA-AT antisense and wild-type plants to assess accumulation of LHC apoproteins for PSI and PSII under Chl deficiency. The amount of D1 was compared with that of LHC apoproteins in extracts of leaf 6. Monospecific antibodies raised against individual LHC apoproteins have

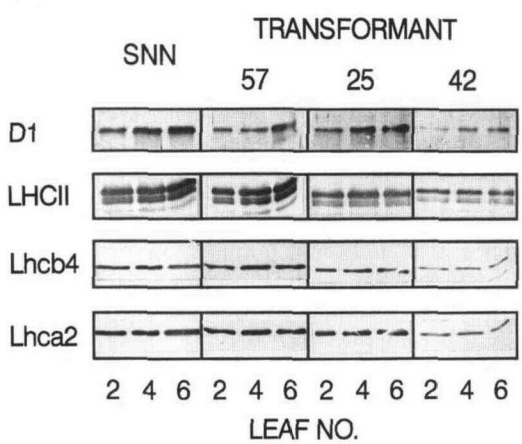

Figure 5. Quantitation of Chl-binding apoproteins throughout leaf development. Levels of the PSII reaction center protein D1, major LHCII, Lhcb4, and Lhca2 were determined in leaf extracts from wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42. Protein extracts described in Figure 4 were subjected to western-blot analyses.

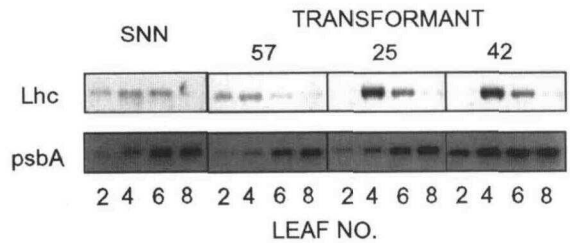

Figure 6. Comparison of steady-state levels of *Lhc* and *psbA* mRNA. Total RNA was extracted from developing leaves 2, 4, 6, and 8 of wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42. In each lane an equal amount of RNA was probed with cDNA encoding LHCII and D1.

previously been used to distinguish LHC subunits of PSI and PSII from spinach and barley (Sigrist and Staehelin, 1994; Król et al., 1995). We also used these antibodies in western blots of the tobacco protein extracts. The observation of polypeptides with expected molecular masses is indicative of specific cross-reaction with heterologous LHC apoproteins of tobacco (Fig. 7). Abundance of the Lhca2 22-kD apoprotein (lower band) was quantified with the CMp antibody. A more intense immune-reacting band was obtained for Lhcb4 (upper band) relative to the Lhcb4-specific antibody (see the Lhcb4 lane). However, the relative intensities of the Lhcb4 bands obtained with the two antibodies were consistent among the various extracts. The

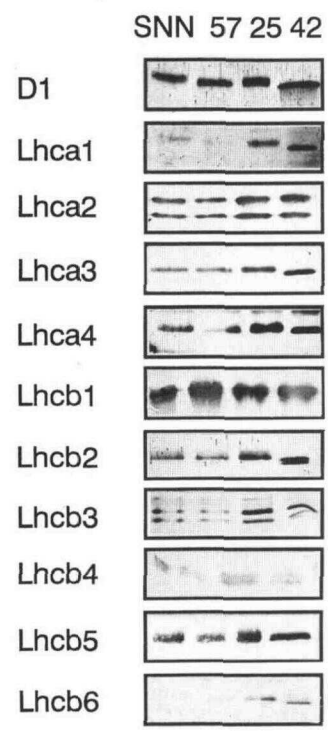

Figure 7. Quantitation of individual LHC apoprotein levels. Protein extracts of leaf 6 from wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42 were adjusted to yield the same staining intensity of the PSII reaction center protein D1. Samples were probed with monoclonal antibodies directed against PSII polypeptides Lhcb1 to Lhcb6 and PSI polypeptides Lhca1 to Lhca4.

Lhcb3 antibody showed cross-reactivity with two additional proteins, in agreement with Król et al. (1995). The intense middle band at 25 kD corresponded in size to Lhcb3. Apart from a few minor immune-reacting bands of high molecular mass obtained with some antibodies, no further cross-reactivity to other LHC apoproteins was observed. As is evident from Figure 7, the LHCII and LHCI apoprotein pattern is very similar in leaves of the wild type and transformant no. 57, but appears somewhat modified in transformants nos. 25 and 42 when related to D1 protein content. In the latter two transformants the apoproteins of Lhcb3 and Lhcb6 are slightly more abundant than in the wild type. In addition, an increase in the content of the Lhca1 and Lhca4 polypeptides in these two transformants was detected.

Since merely the abundance of apoproteins must not be indicative for completely assembled LHCs, we also compared the amounts of Chl associated with pigment-protein complexes of PSII and PSI. Thylakoids from wild-type and transformant leaves were analyzed for abundance of pigment-protein complexes by native polyacrylamide gels. A representative result from a series of experiments is shown in Figure 8. Notably, the Chl *a*/*b* ratios of the various thylakoid samples loaded onto the gels were in a very narrow range around 3.1. Densitometric scanning of pigment-protein complexes revealed very similar LHCII contents (calculated from the sum of the monomeric and trimeric forms of LHCII) and of PSI relative to the respective core antenna complexes even in the most chlorotic transformant (no. 42) relative to the wild type. For comparison, immune-stained major LHCII and the D1 reaction center proteins were also depicted, revealing a similar stoichiometric pattern, as do the solubilized pigment-protein complexes in transformants and wild type.

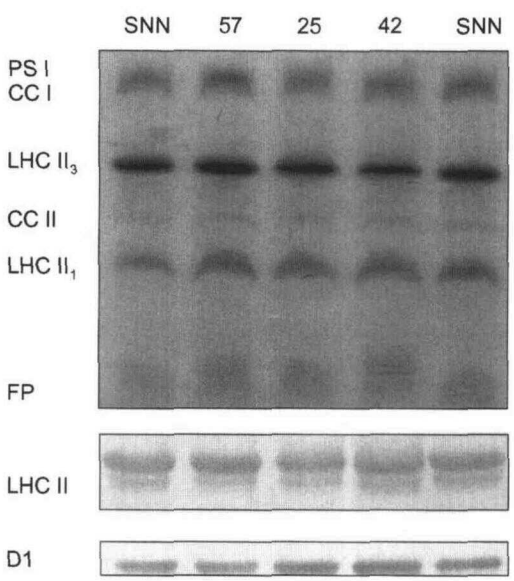

Figure 8. Separation of individual pigment-protein complexes. Upper, Thylakoids from leaves of wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42 solubilized and separated into pigment-protein complexes with mild denaturating PAGE. The gel was not stained. CCI and CCII, Core complex of PSI and PSII, respectively; FP, free pigment; LHC II$_1$ and LHC II$_3$, monomeric and trimeric LHCII, respectively. Middle and lower, Immune-stained quantitation of proteins of the LHCII and CCII bands with antibodies raised against major LHCII and D1.

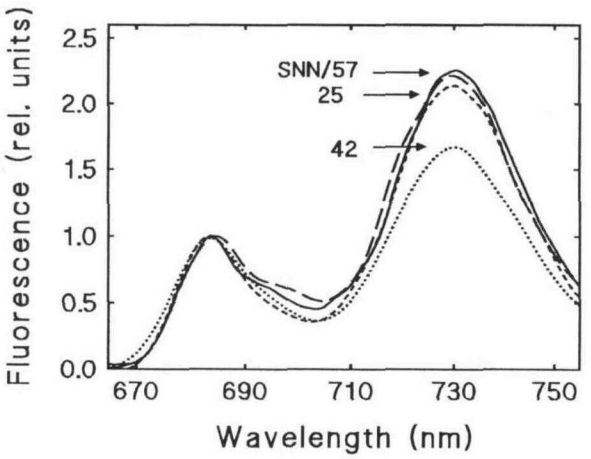

Figure 9. Low-temperature (77 K) Chl fluorescence emission spectra obtained with thylakoids from leaves of wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42. Samples adjusted to a Chl concentration equivalent to 2.5 μg mL^{-1} were excited at 472 nm (Chl *b* excitation). The spectra were normalized to the emission maximum at 685 nm.

Low-Temperature Fluorescence Characteristics Indicate Complete Assembly of Photosynthetic Units

Low-temperature (77 K) fluorescence emission spectra from thylakoids of wild type and transformants reveal three distinct maxima (Fig. 9). The peaks at 685 and 693 nm are generally accepted as emanating from the PSII core protein complexes CP43 and CP47, whereas the 730-nm fluorescence peak is thought to be associated with PSI and its antenna (LHCI). Except for the transformant no. 42, where the maximum at 730 nm is lower relative to the 685-nm band, there are no appreciable differences in the 77 K fluorescence emission ratios between Chl-deficient transformants and the wild type. Moreover, no shift in wavelength of the 730-nm peak was observed. These fluorescence characteristics are taken as evidence that not only PSII units but also PSI units were completed by LHCs in chlorotic transformants.

Chl Deficiency Only Moderately Affects Photosystem Stoichiometry in GSA-AT Transformants

To provide a measure for the antenna sizes of PSII and PSI, the number of Chls per PSII reaction center (calculated by the binding sites of the secondary quinone acceptor) and PSI reaction centers (calculated by the amount of P700) were determined. The number of Chl molecules per PSII and PSI reaction center was largely constant even in GSA-AT antisense plants, except for transformant no. 42, in which the number of Chl molecules per PSI reaction center increased to 130% of the wild-type level (Table II). Taking into account the amount of Chl per leaf area (Fig. 2), we calculated the number of reaction centers per unit area. A

Table II. *Numbers of PSI and PSII reaction centers*

Sixth to eighth leaves of wild-type tobacco (SNN) and various GSA-AT transformants were used. The data are mean values (±SD in parentheses) obtained with two sets of plants. In each set five control plants and five plants of the transformants were individually analyzed.

Parameter	SNN	Transformant		
		57	25	42
Chl per PSI (mol Chl mol^{-1} P700)	441 (30)	444 (41)	474 (47)	556 (61)
PSI per leaf area (μmol P700 m^{-2})	1.11 (0.08)	0.66 (0.06)	0.26 (0.03)	0.10 (0.01)
Chl per PSII (mol Chl mol^{-1} atrazine bound)	702 (59)	698 (74)	720 (89)	714 (102)
PSII per leaf area (μmol atrazine bound m^{-2})	0.70 (0.06)	0.42 (0.05)	0.17 (0.02)	0.078 (0.010)
PSII to PSI ratio	0.63	0.64	0.66	0.79

comparison of the number of wild-type and transformant PSI and PSII reaction centers shows a significant decrease in transgenic plants that roughly parallels the reduction in Chl content. Assuming that the number of secondary quinone acceptor binding sites and P700 centers are proportional to the number of PSII and PSI units, a moderate increase was found in the PSII/PSI ratio in transformant no. 42, but not in transgenic lines no. 25 and 57, compared with the wild type.

GSA-AT Transformants Are Photochemically Competent

To assess effects on photosynthetic efficiency, transformants were analyzed for in vivo Chl fluorescence characteristics and O_2 evolution rates. Figure 10 illustrates maximum quantum yields of PSII deduced from F_v/F_m Chl fluorescence ratios measured on attached leaves of dark-adapted plants. The ratio of 0.81 obtained with wild-type leaves is typical for nonphotoinhibited mature leaves. This

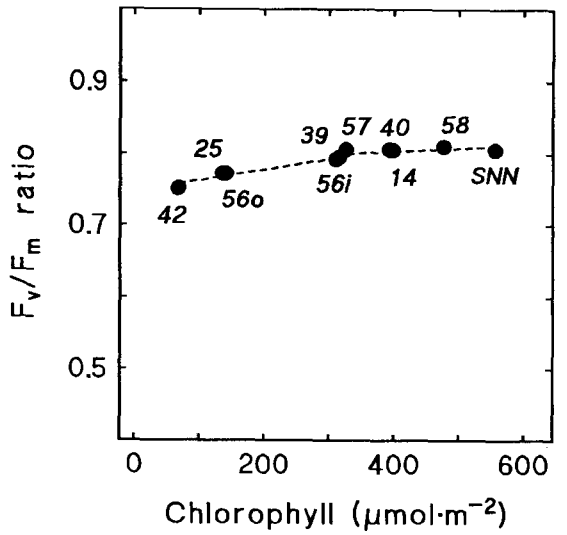

Figure 10. F_v/F_m ratios monitored in 60-min dark-adapted leaves of wild-type tobacco (SNN) and various GSA-AT transformants. Numbers at the data points refer to different transformants, except for transformant no. 56, for which 56i and 56o stand for the inner dark-green and the outer yellow-green leaf tissues, respectively ($n \geq 10$; SD ≤ 8%).

ratio decreased only slightly in the transformants when their Chl contents were reduced below 30% of the wild-type level. The rather small changes in F_v/F_m ratios indicate that GSA-AT antisense plants grown at 70 μmol photons m^{-2} s^{-1} tolerate the strong reduction in Chl content without becoming severely photoinhibited.

To ensure coverage of a representative leaf section of transformants, discs of 9 cm^2 leaf area were used for O_2 evolution measurements. The light-saturated rates of photosynthetic O_2 evolution were considerably lower on a leaf area basis in GSA-AT transformants relative to control plants (Fig. 11A), reflecting the large decreases in the amount of PSII reaction centers (Table II) and Chl (Fig. 2). For comparison, Figure 11B shows the light-response curves of O_2 evolution for thylakoids taken from wild type and transformants on the basis of Chl content; the differences in maximum photosynthetic O_2 evolution rates almost disappear. The similar rates of whole-chain electron transport activity per unit of Chl at saturating light are strongly indicative of a reduction in both antenna Chl and Chl associated with reaction centers. A preferential reduction in the amount of antenna Chl would be expected to result in higher electron transport activities. Furthermore, the slope of the curves (Fig. 11B, inset) was very similar, suggesting a comparable photosynthetic efficiency of PSII reaction centers of chlorotic transformants and wild type.

DISCUSSION

The current understanding of the role of Chl synthesis in the organization of pigment proteins of PSI and PSII has depended on studies with either greening wild-type plants or pigment-deficient mutants. Transgenic plants with progressively reduced Chl-synthesizing capacity due to antisense transcripts for selected enzymes in the tetrapyrrolic metabolic pathway provide a novel approach to examine the functional and regulatory importance of Chl in assembly and stabilization of the photosynthetic machinery. GSA-AT, the final enzyme in the ALA-synthesizing C5 pathway, is an appropriate target to affect the plastidial Chl content. Furthermore, in GSA-AT antisense plants accumulation of Chl intermediates, which may cause photodynamic damage of pigments, lipids, and proteins via production of activated O_2

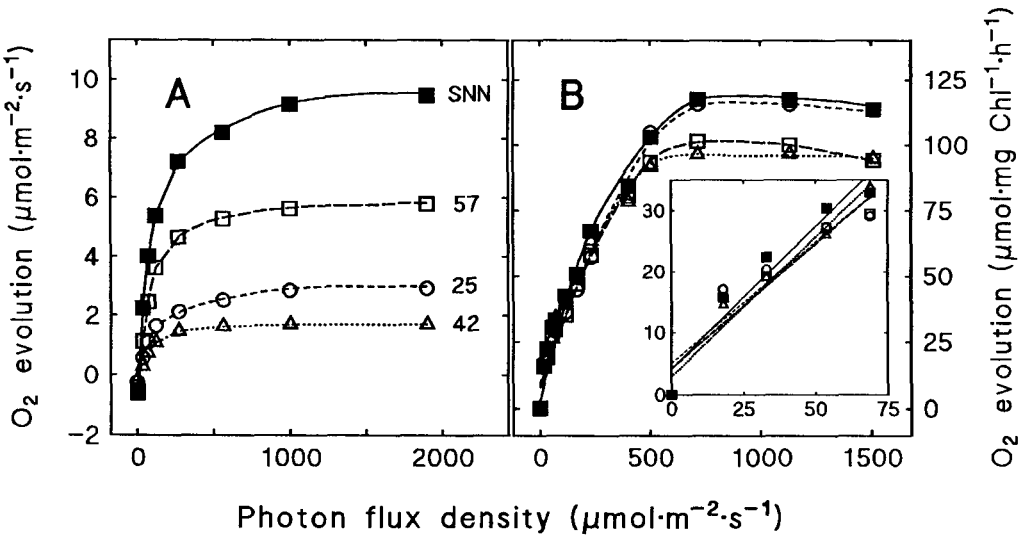

Figure 11. Light response curves for O_2 evolution monitored with leaves (A) or thylakoids (B) of wild-type tobacco (SNN) and the GSA-AT transformants nos. 57, 25, and 42. The inset in B shows the same data in the range of 0 to 70 μmol m^{-2} s^{-1} ($n \geq 5$; SD $\leq$ 15%).

species (Falbel and Staehelin, 1994; Kruse et al., 1995; Härtel et al., 1996b), most probably does not occur.

Transformants showed reduced GSA-AT expression, which resulted in a variable decline in Chl content (Figs. 2 and 3). Comparison of mRNA and protein steady-state levels of ALA-D, GLU-TR, HMBS, and a Mg chelatase subunit with GSA-AT contents confirms the causal connection between the reduction in pigmentation and the reduced GSA-AT-synthesizing capacity in antisense transformants (Figs. 3 and 4). We took advantage of the variable extent of Chl accumulation in GSA-AT transformants and found a remarkably coordinated formation of the photosynthetic apparatus regardless of the amount of Chl that accumulated in leaves. Western-blot analysis using antibodies raised against all known constituents of the photosynthetic antenna showed that virtually all antenna proteins, associated either with PSI or PSII, were present even in the highly chlorotic transformants nos. 25 and 42 (Fig. 7). Studies on plastid development during greening revealed that the accumulation of LHCI apoproteins lags behind that of LHCII apoproteins in thylakoids (Anandan et al., 1993; Dreyfuss and Thornber, 1994). All four Lhca apoproteins appear to be coordinately assembled with the reaction center/core complex of PSI and are usually found in equal stoichiometry within PSI (Anandan et al., 1993; Dreyfuss and Thornber, 1994). The identical 77 K fluorescence emission maxima positions in the wild type and GSA-AT transformants provide evidence for complete assembly of photosynthetic units, particularly of PSI (Fig. 9). The lower fluorescence intensity at 730 nm relative to 685 nm in transformant no. 42 compared with the wild type may reflect the higher PSII/PSI ratio (Table II). Furthermore, Lhca2, a constituent of the LHCI subcomplex LHCI-680, was proposed to function in completing assembly of PSI units (see Jansson, 1994). This apoprotein was immunologically detected at very early developmental stages in transformant no. 42 (leaf 2), conceivably indicating complete assembly of LHCI with no delay. This demonstrates that Chl-deficient transformants are not simply fixed in an early developmental state. The relatively higher increase in Lhca1 and Lhca4 than in Lhca2 and Lhca3 protein levels in transformants when related to their wild-type levels possibly indicates that LHCI apoproteins can be differentially expressed in Chl-deficient GSA-AT antisense plants.

Changes in steady-state LHC protein levels observed by western blotting of total leaf extracts might not necessarily correspond to the amounts of LHC assembled to function in excitation energy transfer to reaction centers. Therefore, to independently confirm the apparent association between changes in pigment content and antenna composition in GSA-AT transformants, solubilized pigment-protein complexes were analyzed by native green gels. The very similar results obtained herein, together with the results of the determination of atrazine-binding sites and P700 content (Table II), clearly show that restriction of Chl synthesis due to expression of GSA-AT antisense RNA does not reduce the light-harvesting antenna size in tobacco under the growth conditions in our study. Moreover, room-temperature fluorescence and O_2 evolution measurements (Figs. 10 and 11) illustrate that photosynthesis operates highly efficiently, even in most bleached transformants. Therefore, we conclude that the steady-state levels of antenna proteins in transformants reflect the amounts of LHC subunits functionally assembled in thylakoid membranes rather than free pools of LHC apoproteins. The number of PSI and PSII reaction centers parallels Chl content (Table II), suggesting that Chl is not only used to stabilize reaction center proteins, but obviously to the same extent also stabilizes LHC proteins. Consequently, the reduced supply of Chl in transformants causes preferential saturation of photosynthetic units, which are, however, reduced in amount.

The preservation of the light-harvesting antenna size in GSA-AT transformants differs from previous studies on structure and organization of photosynthetic units in Chl-

deficient plants. For example, etiolated cucumber cotyledons preferentially accumulate core pigment-protein complexes of PSI and PSII during early stages of greening under conditions of limiting Chl *a* (Shimada et al., 1990). LHCs accumulated after apoproteins of the reaction center/core complex were saturated with Chl *a*. It has additionally been proposed that LHC apoproteins can be degraded during assembly of reaction center/core complexes under conditions of limited Chl biosynthesis (Tanaka et al., 1991). Similarly, limited Chl availability in intermittent-light-grown plants and in Chl *b*-deficient mutants resulted in a decrease in photosynthetic unit size rather than in the number of photosynthetic units (Okabe et al., 1977; Ghirardi et al., 1986; Preiss and Thornber, 1995; Härtel et al., 1996a). Thus, the formation of complete photosynthetic units upon Chl deficiency is hitherto an undescribed phenomenon.

Comparison of steady-state Chl levels and accumulation of Chl-binding proteins reveals a rough quantitative correlation between the accumulation of pigment proteins and their chromophores (Figs. 2, 5, and 7), indicating that synthesis of both constituents is coupled during higher plant chloroplast development. Chl content seems primarily to determine the number and the sequential assembly of antenna complexes, likely by stabilizing apoproteins against protease degradation, as suggested for LHCII (Hoober and Hughes, 1992). Recently, Flachmann and Kühlbrandt (1995) reported a 95% reduction in steady-state *Lhcb* mRNA concentration by antisense RNA, but LHCII accumulated to a normal level in these transgenic plants. The steady-state transcript levels of various *Lhcb* genes appeared not to be rate-limiting for the accumulation of antenna proteins. Reduced LHCII protein turnover upon an adequate Chl supply could explain the results obtained by Flachmann and Kühlbrandt (1995). In GSA-AT antisense transformants, an 88% reduction of Chl content caused an apparent parallel decline in the content of LHCs and reaction centers without reduction of the antenna size per unit. This indicates that inactivation of an early step in Chl synthesis reduces the numbers of both LHCs and reaction centers. Therefore, regulated expression of genes encoding enzymes of the ALA-synthesizing pathway could be an important prerequisite for coordinated stoichiometry of LHCs.

Chl and carotenoid contents showed a strong correlation in photosynthetically active mature leaves independent of the Chl content in transgenic and wild-type plants (Fig. 2). This does not seem spectacular, since both pigment groups are important for stable assembly of pigment-protein complexes. However, if GSA-AT antisense RNA synthesis specifically affects Chl synthesis, considerably higher amounts of carotenoids would be expected to accumulate relative to Chls in transformants. Indeed, the youngest leaves (2 and 4) of the most bleached transformant (no. 42) contain similar carotenoid levels as the wild type on a fresh weight basis (Table I), whereas in older leaves carotenoid levels are counterbalanced with Chls. The simultaneous decrease in carotenoid contents in mature leaves of Chl-deficient transformants may be the result of down-regulated carotenoid synthesis or increased carotenoid turnover, suggesting a regulatory interaction between Chl and carotenoid synthesis. The reduction in the contents of neoxanthin and lutein in parallel with Chl content per unit of leaf area (Fig. 2, C and D) presumably reflects the synchronized association of these pigments with LHC proteins. The tight coupling in stoichiometry of these pigments to Chls has also recently been shown in pigment mutants (Herrin et al., 1992; Plumley and Schmidt, 1995).

It was the aim of this study to analyze total leaf extracts of tobacco wild type and the GSA-AT transformants. Variegation patterns on leaves of transformants nos. 57 and 25 do not detract from our results, since whole leaves were used for measurements of pigment contents, O_2 evolution rates, and Chl fluorescence. Moreover, thylakoid preparations represent the overall leaf plastid spectrum. Therefore, the results obtained with nos. 57 and 25 verify those obtained with the wild type and the uniformly bleached transformant no. 42.

In summary, regulation of GSA-AT synthesis represents a sensitive step in Chl synthesis. Our results unequivocally demonstrate that major decreases in Chl content correlate with a simultaneous reduction of reaction centers and antenna complexes. Thus, the presented findings are consistent with the view that Chl allocation is a primary determinant for assembly of the light-harvesting apparatus. Results independently obtained from different experimental approaches clearly showed completely assembled photosynthetic units even when Chl content was reduced to 12% of wild-type levels. Conceivably, it is this organizational pattern that provides the basis for the stability of the photosynthetic apparatus in Chl-deficient transformants, allowing efficient and regulated light utilization. Therefore, we suggest that under the growth conditions chosen during our study, plants tend to maintain their light-harvesting antenna size even when Chl synthesis is suppressed. It will be interesting to study the consequences of gradually suppressed Chl synthesis on the organization of the photosynthetic apparatus under different light and other environmental conditions. Such experiments are currently in progress in our laboratory.

ACKNOWLEDGMENTS

We acknowledge Prof. Marvin Smith (Brigham Young University, Provo, UT) for critical reading of the manuscript. Prof. Gernot Renger (Max-Volmer Institute, Technical University, Berlin, Germany) is acknowledged for the generous gift of labeled atrazine. Monoclonal antibodies were generously provided by Dr. Stefan Jansson (Umea University, Sweden) for Lhca1, Lhca3, and Lhca4, and Prof. L. Andrew Staehelin (University of Colorado, Boulder) for Lhcb1, Lhcb2, Lhcb3, Lhcb5, and Lhcb6. Polyclonal antibodies were kindly provided by Dr. Alison Smith (Department of Plant Science, Cambridge University, UK) for HMBS, Prof. Hans-Jörg Schneider-Poetsch (Botanical Institute, Cologne, Germany) for ALA-D, Prof. Diter von Wettstein (Carlsberg Laboratory, Copenhagen, Denmark) for Lhca2 + Lhcb4, Prof. Joseph Hirschberg (Hebrew University, Jerusalem, Israel) for D1 protein, and Prof. Klaus Kloppstech (Institute for Botany, Hannover, Germany) for LHCII.

Received September 26, 1996; accepted December 27, 1996.

LITERATURE CITED

Allen KD, Staehelin LA (1991) Resolution of 16 to 20 chlorophyll-protein complexes using a low ionic strength native green gel system. Anal Biochem **194:** 214–222

Anandan S, Morishige DT, Thornber JP (1993) Light-induced biogenesis of light-harvesting complex I (LHCI) during chloroplast development in barley. Plant Physiol **101:** 227–236

Andersson B, Anderson JM, Ryrie IJ (1982) Transbilayer organization of the chlorophyll-proteins of spinach thylakoids. Eur J Biochem **123:** 465–472

Apel K, Kloppstech K (1980) The effect of light on the biosynthesis of the light-harvesting chlorophyll *a/b*-binding-protein: evidence for the requirement of chlorophyll *a* for the stabilization of the apoprotein. Planta **150:** 426–430

Argyroundi-Akoyunoglou JH, Akoyunoglou A, Kalosakas K, Akoyunoglou G (1982) Reorganization of the photosystem II unit in developing thylakoids of higher plants after transfer to darkness. Plant Physiol **70:** 1242–1248

Beale SI, Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organisms. *In* HA Dailey, ed, Biosynthesis of Heme and Chlorophyll. McGraw-Hill, New York, pp 287–391

Bougri O, Grimm B (1996) Members of a low-copy number gene family encoding glutamyl-tRNA reductase are differentially expressed in barley. Plant J **9:** 867–878

Camm EL, Green BR (1982) The effects of cations and trypsin on extraction of chlorophyll-protein complexes by octyl glucoside. Arch Biochem Biophys **214:** 563–572

Chomczinsky P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156–159

Dreyfuss BW, Thornber JP (1994) Organization of the light-harvesting complex of photosystem I and its assembly during plastid development. Plant Physiol **106:** 841–848

Falbel TG, Staehelin LA (1994) Characterization of a family of chlorophyll-deficient wheat (*Triticum*) and barley (*Hordeum vulgare*) mutants with defects in the magnesium-insertion step of chlorophyll biosynthesis. Plant Physiol **104:** 639–648

Flachmann R, Kühlbrandt W (1995) Accumulation of plant antenna complexes is regulated by post-transcriptional mechanisms in tobacco. Plant Cell **7:** 149–160

Ghirardi ML, McCauley SW, Melis A (1986) Photochemical apparatus organization in the thylakoid membrane of *Hordeum vulgare* wild type and chlorophyll b-less chlorina f2 mutant. Biochim Biophys Acta **851:** 331–339

Grimm B (1990) Primary structure of a key enzyme in plant tetrapyrrole synthesis: glutamate 1-semialdehyde aminotransferase. Proc Natl Acad Sci USA **87:** 4169–4173

Härtel H, Haseloff RF, Walter G, Rank B (1993) Photoinduced damage in leaf segments of wheat (*Triticum aestivum* L.) and lettuce (*Lactuca sativa* L.) treated with 5-aminolevulinic acid. II. Characterization of photodynamic damage by means of delayed chlorophyll fluorescence and P700 photooxidation. J Plant Physiol **142:** 237–243

Härtel H, Lokstein H, Grimm B, Rank B (1996a) Kinetic studies on the xanthophyll cycle in barley leaves. Influence of antenna size and relations to nonphotochemical chlorophyll fluorescence quenching. Plant Physiol **110:** 471–482

Härtel H, Walter G, Haseloff RF, Hoffmann P, Renger G (1996b) Photochemical and non-photochemical energy dissipation in response to 5-aminolaevulinic acid-induced photosensitization of green leaves of wheat (*Triticum aestivum* L.) and lettuce (*Lactuca sativa* L.). J Photochem Photobiol B Biol **32:** 115–126

He Z-H, Li J, Sundqvist C, Timko MP (1994) Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.). Plant Physiol **106:** 537–546

Herrin DL, Battey JF, Greer K, Schmidt GW (1992) Regulation of chlorophyll apoprotein expression and accumulation. J Biol Chem **267:** 8260–8269

Hiyama T, Ke B (1972) Difference spectra and extinction coefficients of P-700. Biochim Biophys Acta **267:** 160–171

Höfgen R, Axelsen KA, Kannangara CG, Schüttke I, Pohlenz H-D, Willmitzer L, Grimm B, von Wettstein D (1994) A visible marker for antisense mRNA expression in plants: inhibition of chlorophyll synthesis with a glutamate 1-semialdehyde aminotransferase antisense gene. Proc Natl Acad Sci USA **91:** 1726–1730

Hoober JK, Hughes MJ (1992) Purification and characterization of a membrane-bound protease from *Chlamydomonas reinhardtii.* Plant Physiol **99:** 932–937

Jansson S (1994) The light-harvesting chlorophyll *a/b* binding proteins. Biochim Biophys Acta **1184:** 1–19

Jansson S, Pichersky E, Bassi R, Green BR, Ikeuchi M, Melis A, Simpson DJ, Spangfort M, Staehelin LA, Thornber JP (1992) A nomenclature for the genes encoding the chlorophyll *a/b*-binding proteins of higher plants. Plant Mol Biol Rep **10:** 242–253

Kannangara CG, Andersen RV, Poutoppidan B, Willows R, von Wettstein D (1994) Enzymic and mechanistic studies on the conversion of glutamate to 5-aminolaevulinate. *In* DJ Chadwick, K Ackvill, eds, The Biosynthesis of the Tetrapyrrole Pigments. John Wiley & Sons, Chichester, UK, pp 3–25

Kim J, Eichacker LA, Rüdiger W, Mullet JE (1994) Chlorophyll regulates accumulation of the plastid-encoded chlorophyll proteins P700 and D1 by increasing apoprotein stability. Plant Physiol **104:** 907–916

Klein PR, Gamble PE, Mullet JE (1988) Light-dependent accumulation of radiolabeled plastid-encoded chlorophyll *a*-apoproteins requires chlorophyll *a*. I. Analysis of chlorophyll-deficient mutants and phytochrome involvement. Plant Physiol **88:** 1246–1256

Knoetzel J, Simpson D (1991) Expression and organization of antenna proteins in the light-sensitive and temperature-sensitive barley mutant *chlorina*-^{104}. Planta **185:** 111–123

Król M, Spangfort MD, Huner NPA, Öquist G, Gustafsson P, Jansson S (1995) Chlorophyll *a/b*-binding proteins, pigment conversions, and early light-induced proteins in a chlorophyll *b*-less barley mutant. Plant Physiol **107:** 873–883

Kruse E, Mock H-P, Grimm B (1995) Reduction of coproporphyrinogen oxidase level by antisense RNA synthesis leads to deregulated gene expression of plastid proteins and affects the oxidative defense system. EMBO J **14:** 3712–3720

Kühlbrandt W, Wang DA, Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature **367:** 614–621

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. Methods Enzymol **148:** 350–382

Marsho TV, Kok B (1980) P700 detection. Methods Enzymol **69:** 280–289

Mathis JN, Burkey KO (1989) Light intensity regulates the accumulation of the major light-harvesting chlorophyll-protein in greening seedlings. Plant Physiol **90:** 560–566

Mullet JE (1988) Chloroplast development and gene expression. Annu Rev Plant Physiol Plant Mol Biol **39:** 475–502

Okabe K, Schmid GH, Straub J (1977) Genetic characterization and high efficiency photosynthesis of an aurea mutant of tobacco. Plant Physiol **60:** 150–156

Paulsen H, Rümler U, Rüdiger W (1990) Reconstitution of pigment-containing complexes from light-harvesting chlorophyll *a/b*-binding protein overexpressed in *Escherichia coli.* Planta **181:** 204–211

Plumley FG, Schmidt GW (1987) Reconstitution of chlorophyll *a/b* light-harvesting complexes: xanthophyll-dependent assembly and energy transfer. Proc Natl Acad Sci USA **84:** 146–150

Plumley FG, Schmidt GW (1995) Light-harvesting chlorophyll *a/b* complexes: interdependent pigment synthesis and protein assembly. Plant Cell **7:** 689–704

Preiss S, Thornber JP (1995) Stability of the apoproteins of light-harvesting complex I and II during biogenesis of thylakoids in the chlorophyll *b*-less barley mutant *chlorina* f2. Plant Physiol **107:** 709–717

Shimada Y, Tanaka A, Tanaka Y, Takabe T, Tsuji H (1990) Formation of chlorophyll-protein complexes during greening. I. Distribution of newly synthesized chlorophyll. Plant Cell Physiol **31:** 639–647

Sigrist M, Staehelin LA (1994) Appearance of type 1, 2, and 3 light-harvesting complex II and light-harvesting complex I proteins during light-induced greening of barley (*Hordeum vulgare*) etioplasts. Plant Physiol **104:** 135–145

Tanaka A, Yamamoto Y, Tsuji H (1991) Formation of chlorophyll-protein complexes during greening. II. Redistribution of chlorophyll among proteins. Plant Cell Physiol **32:** 195–204

Tischer W, Strotmann H (1977) Relationships between inhibitor binding by chloroplasts and inhibition of photosynthetic electron transport. Biochim Biophys Acta **460:** 113–125

von Wettstein D, Gough S, Kannangara CG (1995) Chlorophyll biosynthesis. Plant Cell **7:** 1039–1057