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Antisense *HEMA1* RNA Expression Inhibits Heme and Chlorophyll Biosynthesis in Arabidopsis[1]

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5-Aminolevulinic acid (ALA) is a precursor in the biosynthesis of tetrapyrroles including chlorophylls and heme. The formation of ALA involves two enzymatic steps which take place in the chloroplast in plants. The first enzyme, glutamyl-tRNA reductase, and the second enzyme, glutamate-1-semialdehyde-2,1-aminomutase, are encoded by the nuclear *HEMA* and *GSA* genes, respectively. To assess the significance of the *HEMA* gene for chlorophyll and heme synthesis, transgenic Arabidopsis plants that expressed antisense *HEMA1* mRNA from the constitutive cauliflower mosaic virus 35S promoter were generated. These plants exhibited varying degrees of chlorophyll deficiency, ranging from patchy yellow to total yellow. Analysis indicated that these plants had decreased levels of chlorophyll, non-covalently bound hemes, and ALA; their levels were proportional to the level of glutamyl-tRNA reductase expression and were inversely related to the levels of antisense *HEMA* transcripts. Plants that lacked chlorophyll failed to survive under normal growth conditions, indicating that *HEMA* gene expression is essential for growth.

Porphyrin compounds such as chlorophyll and heme play vital roles in plant metabolism. The porphyrin ring system is derived from 5-aminolevulinic acid (ALA). In plants ALA is formed from the five-carbon skeleton of Glu in two steps known as the C_5 pathway (Beale, 1978; von Wettstein et al., 1995; Kumar et al., 1996b). All of the components required for such a conversion are located in the chloroplasts. The initial metabolite of the C_5 pathway is chloroplast-specific glutamyl-tRNA^{Glu} (Schön et al., 1986). In the first step, glutamyl-tRNA reductase (GluTR) reduces the glutamyl moiety of Glu-tRNA to Glu-1-semialdehyde (GSA). In the subsequent reaction, GSA is converted to ALA by GSA-2,1-aminomutase (GSA-AM). GluTR and GSA-AM are encoded by the nuclear *HEMA* and *GSA* genes. In Arabidopsis and other plants two *HEMA* genes exist (Ilag et al., 1994; Kumar et al., 1996a; Tanaka et al., 1996; Grimm, 1998; Sangwan and O'Brian, 1999). The *HEMA1* gene is regulated by light and expressed in all parts of the plant, while the expression of the *HEMA2* gene was only found in roots and flowers in a light-independent fashion. The *HEMA1* and *HEMA2* genes are very similar (79% at the nucleotide level in the coding region and differ in the 5′- and 3′-untranslated regions), while the gene products also show high similarity (83%).

Although it is widely accepted that all ALA in plants is formed in the C_5 pathway, the presence of the C_4 pathway in plants has been implied (Beale, 1978). In this biosynthetic route, which operates in animal mitochondria and in yeast, ALA is generated by ALA synthase from Gly and succinyl coenzyme A (May et al., 1990). ALA synthase-like activity has been monitored in greening potato skin (Ramaswamy and Nair, 1973) and soybean callus (Meller and Gassman, 1982); however, the enzyme was never characterized from plants. Other lines of evidence, e.g. photodestruction of chloroplasts (Thomsen et al., 1993), mutations affecting the tRNA^{Glu} (Hess et al., 1992), and inhibition of GSA-AT activity by gabaculin (Flint, 1984), also support the C_4 pathway of ALA biosynthesis in plants. We describe studies of antisense *HEMA1* Arabidopsis plants that unequivocally demonstrate the involvement of GluTR in ALA formation.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli strain DH$_5\alpha$ was used for routine recombinant DNA work. *Agrobacterium tumefaciens* strains EHA101 (for hypocotyl infection) and GV 3101 (for vacuum infiltration) were used in the generation of Arabidopsis transgenic lines.

Construction of the *HEMA* Antisense Vector

A *Nco*I site was created by site-directed mutagenesis at the translation start site in *HEMA1* cDNA (Sambrook et al., 1989). After confirming the sequence, the entire coding region of the *HEMA1* cDNA was released by digestion with *Nco*I and *Sph*I restriction endonucleases. This fragment was blunt-ended with Klenow DNA polymerase and ligated to *Nco*I/*Nru*I-digested, blunt-ended pRTL2-GUS plasmid. Transformants were analyzed for the constructs containing the *HEMA* nucleotide sequence in the antisense orientation downstream of the cauliflower mosaic virus 35S promoter. From the confirmed construct, a DNA fragment containing the cauliflower mosaic virus 35S promoter, *HEMA* cDNA (in antisense orientation), and the terminator was isolated as a cassette and ligated to *Sal*I-digested, blunt-ended binary vector pCIT20. Plasmids pRTL2-GUS and pCIT20 were from Dr. X.-W. Deng's laboratory (Yale University).

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The resultant construct was transformed into the *A. tumefaciens* strains EHA101 and GV3101.

Plant Growth and Transformation

Hypocotyl Infection

Arabidopsis (ecotype Columbia) plants were grown from surface-sterilized seeds on sterile Murashige and Skoog agar medium containing 0.1% (w/v) Suc and 0.1 g/L myo-inositol under standard conditions (22°C, 60% relative humidity with a regimen of 16 h of 90 μE m^{-2} s^{-1} white light and 8-h dark day cycle). Hypocotyls were isolated from 10-d-old seedlings and hand-prepared for *A. tumefaciens* infection as previously described (Akama et al., 1992). Growth conditions involved in generating well-differentiated plants from the infected hypocotyls using root- and growth-inducing media were as previously described (Akama et al., 1992).

Vacuum Infiltration

Arabidopsis seeds were grown in vermiculite under the standard growth conditions. When primary inflorescence shoots attained a reasonable height (2–3 cm), they were cut off to generate multiple secondary inflorescences (which took about 7–10 d). At this stage, plants were used for *A. tumefaciens* infection by vacuum infiltration as described previously (Berthold et al., 1993).

DNA Isolation and Analysis

Total DNA (5 μg) isolated from the 2-week-old transgenic and control Arabidopsis plants was digested with *Bam*HI, and the restricted fragments were separated on a 0.8% (w/v) agarose gel. Transfer of restriction fragments to a nitrocellulose membrane, prehybridization, hybridization, and washing of the membrane were performed under stringent conditions (Sambrook et al., 1989). The probe (a 600-bp *Nhe*I fragment from the *HEMA1* cDNA) was labeled with [α-^{32}P]dATP as described previously (Sambrook et al., 1989).

RNA Extractions and Northern-Blot Analysis

Total RNA was isolated (Ilag et al., 1994) from 2-week-old transgenic and control Arabidopsis plants. RNA (20 μg) was heat-denatured (85°C for 2 min) in the presence of formamide (50%, w/v) and separated on a denaturing 1.2% (w/v) agarose gel as described previously (Sambrook et al., 1989). Equal loading of RNA in northern-blot analysis was confirmed by hybridization with a pea 18S rDNA fragment. Prehybridization, hybridization, and washing conditions of the membranes were described above. To monitor the expression levels of endogenous genes, respective DNA fragments were labeled with [α-^{32}P]dATP using random hexanucleotide priming (Sambrook et al., 1989). We used strand-specific RNA probes prepared from *HEMA* cDNA clone using T3 and T7 RNA polymerase for the estimation of sense and antisense expression of the *HEMA* gene in these plants (Sambrook et al., 1989).

Protein Extractions and Western-Blot Analysis

Plants were ground in liquid nitrogen and suspended (100 μL/100 mg) in extraction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 50 mM β-mercaptoethanol, 0.5% (w/v) Triton X-100, 0.2 mM phenylmethylsulfonylfluoride, 1 μg/mL leupeptin, and 1 μg/mL apoprotein. After a brief vortexing, the suspension was centrifuged at 13,000*g* for 10 min and the supernatant collected. The protein in the supernatant was measured with the dye-binding assay (Bradford, 1976). Separation of proteins by SDS-PAGE and blotting was performed as described previously (Sambrook et al., 1989). Blots were incubated for 1 h with respective antibodies (1:1,000 dilution) in a solution containing 50 mM Tris-HCl (pH 10.2), 150 mM NaCl, 1% (w/v) bovine serum albumin, and 0.1% (w/v) Triton X-100. The blots were washed three times (for 5 min each) with the same solution without bovine serum albumin. The western-blot analysis of protochlorophyllide oxidoreductase (Por) proteins was performed as previously described (Armstrong et al., 1995).

Biochemical Analysis

Chlorophyll Estimation

Chlorophyll pigment was extracted into 80% (w/v) aqueous acetone and the total chlorophyll (chlorophyll *a*+*b*) was estimated as described previously (Lichtenthaler, 1987).

Quantitation of Heme

Non-covalently bound hemes (protoheme and heme *a*) were extracted by acid acetone and purified on a DEAE-Sepharose column as reported previously (Weinstein and Beale, 1984). Pyridine (final concentration, 25%) and NaOH (final concentration, 0.1 N) were added to the processed eluate (to convert the protoheme and heme *a* in the eluate to pyridine homochrome), followed by few crystals of sodium dithionite (to reduce the pyridine homochromes), as described previously (Stillman and Gassman, 1978). The absorption spectrum of the reduced pyridine homochrome was recorded between wavelengths of 400 to 600 nm with a UV/VIS spectrometer (Lambda 2, Perkin-Elmer, Foster City, CA).

ALA Determination

ALA was extracted from Arabidopsis plants, purified, and measured colorimetrically, as described previously (Weinstein and Beale, 1985). However, prior to the extraction, plants were incubated for 6 h in Murashige and Skoog medium containing levulinic acid (100 mM).

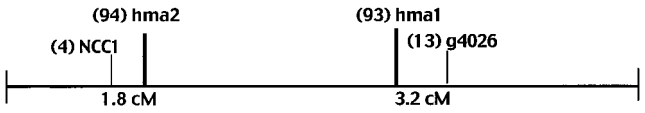

Figure 1. Schematic representation of the location of *HEMA1* (hma1) and *HEMA2* (hma2) genes on the Arabidopsis chromosome I. The distance (in centiMorgans [cM]) and the markers nearest to these genes are shown. Numbers in parentheses indicate the marker identification.

RESULTS

HEMA1 and *HEMA2* Map to Chromosome I

Several restriction enzymes were used to reveal the polymorphisms between pure Arabidopsis ecotypes Columbia and Landsberg *erecta* for *HEMA1* and *HEMA2* genes (Lister and Dean, 1993). The 3′-untranslated DNA fragments of *HEMA1* and *HEMA2* genes were used as gene-specific probes. After identifying the RFLP pattern for *HEMA1* and *HEMA2* genes, the genomic DNA isolated from 29 inbred lines derived from a cross of Columbia and Landsberg *erecta* ecotypes was digested with *Eco*RV (for *HEMA1*) and *Bgl*II (for *HEMA2*) and Southern-blot analysis was carried out with gene-specific probes as described above. The data on segregation of polymorphisms in these lines were analyzed with the Mapmaker program (Lander et al., 1987), which placed the *HEMA1* and *HEMA2* genes to chromosome I in Arabidopsis (Fig. 1).

Generation and Characterization of Antisense Plants

Infection of Arabidopsis hypocotyls with the *A. tumefaciens* strain EHA101 harboring the binary pCIT20-*HEMA* antisense construct resulted in the growth of several hygromycin-resistant shoots. Plantlets from these shoots were generated by transferring onto root-inducing medium followed by growth-inducing medium, as described previously (Akama et al., 1992). Similarly, seeds obtained from Arabidopsis plants vacuum infiltrated with transformed *A. tumefaciens* were plated on Murashige and Skoog medium containing Suc (3%, w/v) and hygromycin (20 μg/mL) to select hygromycin-resistant growth. The transformation efficiency by this protocol was found to be less than 0.1%. However, all the transgenic plants characterized in this study are derived from hypocotyl infection. From a total of 25 transformants that were established for hygromycin-resistant growth, four lines (2-1B2, 2-10PG1, 2-10PG2, and 17-10) exhibiting varying degrees of chlorophyll deficiency were selected for analysis. The phenotypes of these plants are shown in Figure 2.

Correlation of Chlorophyll and Heme Content

As a first step to demonstrate the effect of the expression of antisense *HEMA1* RNA, the levels of chlorophyll and non-covalently bound hemes were measured in the selected four transformants. All of the transgenic plants showed reduced levels of total chlorophyll (23%–82%) compared with the control plants (Fig. 3). Similarly, protoheme content was also lowered (22%–60%) in these plants (Fig. 3). We observed that the levels of chlorophyll and protoheme were proportionally decreased.

The dithionite-reduced pyridine homochrome spectra showed two absorption peaks (data not shown) around 418 and 557 nm. The authentic protoheme when processed similarly also gave two peaks at 418 and 557 nm. Furthermore, it was previously reported that the protoheme purified from the maize seedlings showed two peaks at 418 and 557 nm (Schneegurt and Beale, 1986). Therefore, it was concluded that the protocol used in the present study measures mostly protoheme. However, heme *a* could not be measured satisfactorily as we did not detect peaks at 429 nm and 588 nm as shown previously (Schneegurt and Beale, 1986).

Reduced ALA Levels in the Transformants

To determine if the decreased concentrations of chlorophyll and non-covalently bound hemes in the transgenic plants reflect the amount of ALA synthesis, we measured the level of ALA. Plants treated with levulinic acid showed detectable levels of ALA. As in the case of chlorophyll and

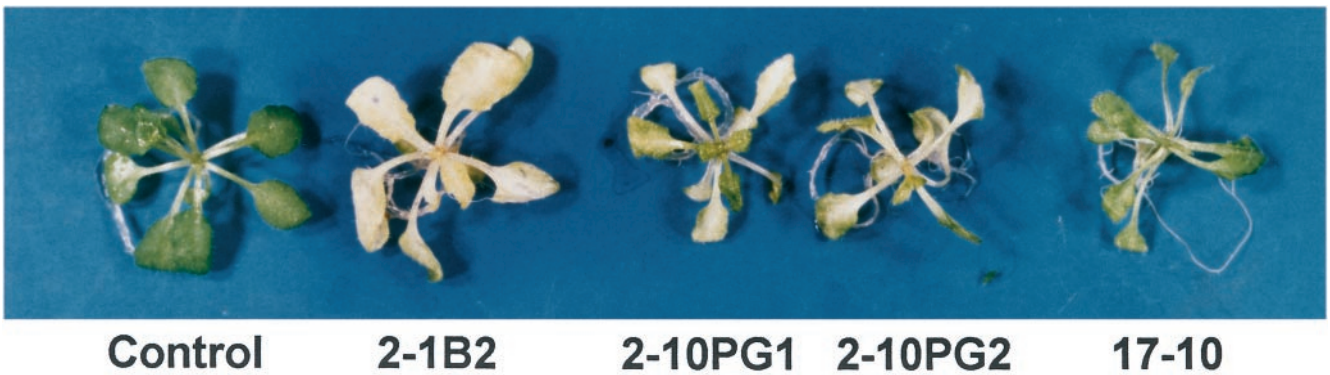

Figure 2. Phenotypes of the selected 3-week-old transgenic Arabidopsis plants. A control plant and four transgenic plants (2-1B2, 2-10PG1, 2-10PG2, and 17-10) representing four different lines exhibiting varying degrees of chlorophyll deficiency are shown. The 2-B12 line represents the severe phenotype and the 17-10 line shows negligible loss of chlorophyll. The other lines (2-10PG1 and 2-10PG2) show intermediary effects of antisense HEMA RNA expression.

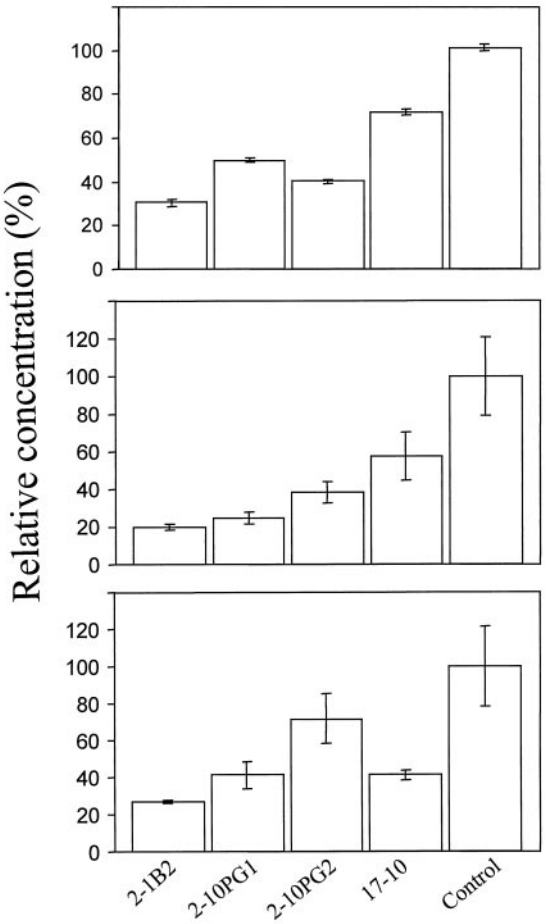

Figure 3. Chlorophyll (top), protoheme (middle), and ALA (bottom) levels in 2-week-old Arabidopsis transgenic lines. The amounts are expressed relative to the amounts present in control plants (taken as 100%). Data are the means of three replicates; SES are shown.

hemes, plant line 2-1B2 accumulated the least amount of ALA. The levels of ALA in the tested plants ranged from 21% to 56% compared with the control (Fig. 3).

Nucleic Acid Analysis of Transgenic Plants

DNA Analysis

Based on the restriction map of the pCIT20-*HEMA* antisense construct it was expected that digestion with *Bam*HI would release a 600-bp fragment from the transgenic plant DNA and not from the control (Fig. 4). However, such an analysis with the transgenic plant DNA yielded not only the 600-bp fragment but also multiple fragments, suggesting that one or multiple copies of the antisense *HEMA1* gene were integrated into the different locations of the chromosome. The Southern-blot analysis also showed hybridization signals in all the transgenic lines that coincided with the *Bam*HI restriction pattern in the control plant (Fig. 4). This result implied that the native *HEMA* gene in the transgenic lines is intact and the phenotype in these plants did not arise due to the disruption of the wild-type gene.

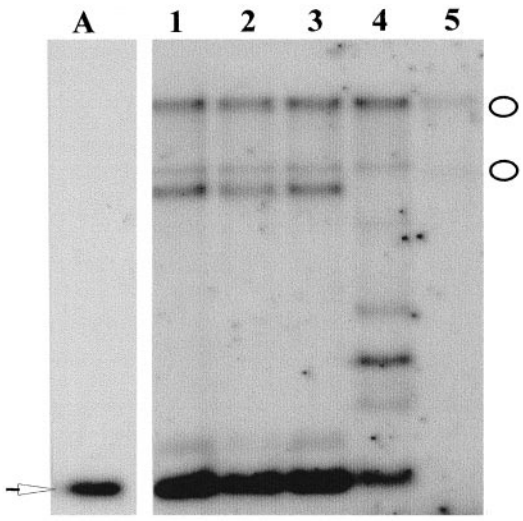

Figure 4. DNA analysis of the transgenic Arabidopsis lines. The *Bam*HI-digested restriction fragments from the pCIT20-*HEMA* antisense construct (lane A) and from the Arabidopsis plants (lanes 1, 2-1B2; 2, 2-10PG1; 3, 2-10PG2; 4, 17-10; and 5, control plant) were size-fractionated by agarose electrophoresis and probed with the [α-^{32}P]dATP labeled 600-bp *Nhe*I *HEMA* fragment. The white arrow shows the fragment in the plasmid digest and in the transgenic lines, indicating the integration of the fragment from the construct into the genome of the transgenic plants. The white ovals show the positions of the hybridization fragments in the control plant and in the transgenic plants, suggesting that the native *HEMA* gene in these transgenic plants is not disrupted by the transformation.

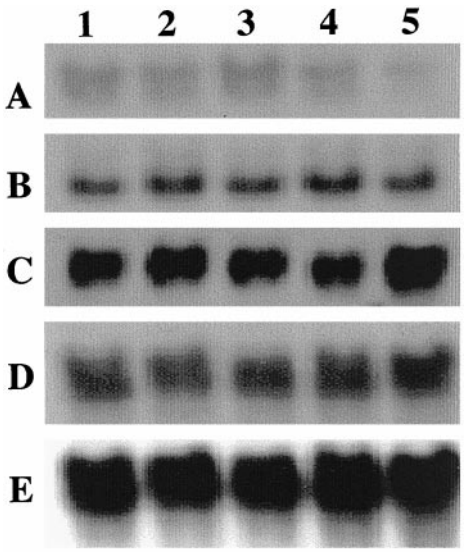

Figure 5. RNA analysis of the transgenic Arabidopsis lines expressing antisense *HEMA1* RNA. Total RNA (20 μg) was isolated from 2-week-old Arabidopsis plants and blotted onto a nitrocellulose membrane as described in "Materials and Methods." Lanes indicate the selected lines: 1, 2-1B2; 2, 2-10PG1; 3, 2-10PG2; 4, 17-10; and 5, control plant. Probes are *HEMA1* cDNA sense strand (A), *HEMA1* cDNA antisense strand (B), *GSA* cDNA (C), small subunit of Rubisco (D), and 18S rDNA (E).

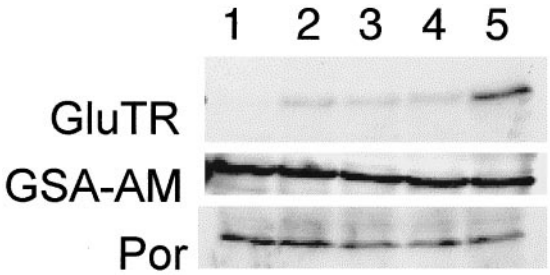

Figure 6. Western-blot analysis of the transgenic Arabidopsis lines expressing antisense *HEMA1* RNA. Lane 1, 2-1B2; lane 2, 2-10PG1; lane 3, 2-10PG2; lane 4, 17-10; and lane 5, control plant.

RNA Analysis

To examine if the observed ALA, chlorophyll, and heme levels of the selected transgenic Arabidopsis plants were due to the inhibition of *HEMA* mRNA expression by antisense mRNA, the steady-state *HEMA* mRNA, and the antisense *HEMA1* mRNA levels were analyzed. All transgenic lines showed an equal amount of the endogenous *HEMA* mRNA levels. However, these lines expressed different levels of antisense mRNA (Fig. 5). The transgenic line expressing the severe phenotype (2-1B2) contained an almost 4-fold elevated level of antisense transcript. Interestingly, the transgenic line (17-10) that did not exhibit phenotypic variation showed a 2-fold increase of antisense transcript compared with the control plant. Thus, there is no strict correlation of the antisense transcript level (as measured by northern blot) with the observed phenotype. The *GSA* gene transcript levels were similar in all the transgenic lines.

Antisense *HEMA1* RNA Expression Lowers GluTR Levels

The GluTR protein level in the extracts was monitored by western-blot analysis using polyclonal anti-GluTR antibodies. These antibodies recognized a 53-kD protein; in the transgenic plants the amount GluTR protein was reduced (Fig. 6). The transgenic line with the most severe phenotype (2-1B2) contained only 1% of the amount found in control plants. To determine if this reduction is specific to the *HEMA* gene product GluTR, we analyzed the expression of Glu-1-semialdehyde-2,1-aminomutase and protochlorophyllide oxidoreductase by western blots using respective antibodies. We observed that all transgenic lines expressed GSA-AM and Por proteins as much as in they were expressed in control plants. This indicates that the *HEMA* antisense RNA specifically suppresses the GluTR expression.

Results obtained by this analysis establish that the expression of antisense *HEMA1* RNA suppresses the formation of ALA, resulting in the reduction of chlorophyll and heme levels in Arabidopsis. This is the first direct demonstration of the vital role of GluTR expression in higher plants.

DISCUSSION

The C_5-Pathway Is Indispensable for Plant Survival

Transgenic Arabidopsis plants expressing antisense *HEMA1* mRNA were generated to monitor the significance of the *HEMA* gene in the formation of ALA. The coding region of the *HEMA1* gene was used to construct the antisense mRNA expression vector. As the *HEMA1* and *HEMA2* genes have extensive similarity at the nucleotide level in the coding region, an antisense construct made from either *HEMA1* cDNA was expected to block the translation of both *HEMA1* and *HEMA2* mRNAs. Some of the transgenics showed chlorophyll deficiency, ranging from patchy yellow to total yellow. We also observed that plantlets that completely lacked chlorophyll failed to survive under the growth conditions described. Attempts to rescue these plants by growing them in either low light or supplementing the medium with varying concentrations of ALA failed. An earlier study carried out with transgenic tobacco plants expressing antisense *GSA* mRNA also showed that plants with reduced chlorophyll levels failed to survive (Höfgen et al., 1994). These observations suggest that suppression of the enzymes of the C_5 pathway affect the growth of the plant. In addition, an analysis of chlorophyll-deficient Arabidopsis mutants revealed plants with lesions in steps of chlorophyll synthesis beyond the point of ALA formation (Runge et al., 1995); the fact that none were found in the C_5 pathway underscores the vital nature of ALA biosynthesis in plants.

The Antisense *HEMA1* Phenotype Is Variable

All of the selected transgenic plants expressed different levels of antisense *HEMA1* mRNA. The antisense RNA is thought to form a duplex with the endogenous *HEMA* mRNA and thus to prevent gene expression. As these duplexes are substrates for double-strand-specific RNases, the detection of either sense or antisense mRNAs in transgenic plants is difficult (e.g. Zrenner et al., 1993; Höfgen et al., 1994). However, we detected both antisense and sense mRNAs in the transgenic plants, suggesting that this was either due to sluggish double-stranded RNA-specific RNase activity or a rapid production of the sense and antisense mRNAs that surpasses the RNase activity. The transgenic plant with the weakest phenotype (17-10) was found to contain several copies of the antisense gene (Fig. 4). Nevertheless, the amount of antisense transcript present in this line was not proportional. This phenomenon has been observed in several instances and could be due to silencing phenomena or position effects (Tabler, 1993). On the other hand, although line 2-1B2 showed fewer copies of antisense *HEMA1* (as judged by Southern blots), the severe phenotype observed was due to the expression of significant amounts of *HEMA* antisense RNA reducing the GluTR level (Fig. 6).

While probing the antisense transcripts with the sense RNA probe, we also noticed a hybridization signal in the control plants. This hybridization is either from the minute quantities of antisense probes that are made by aberrant

priming process during the probe preparation (Sambrook et al., 1989) or, more interestingly, it may due to the naturally occurring antisense RNA in the wild-type plants. Such complementary RNA species, although not widely reported to be involved in gene regulation in plants, have been described; e.g. there is evidence for naturally occurring antisense RNA of an α-amylase gene in barley (Rogers, 1988).

While there is a slight difference between RNA and protein expression values, it is most satisfying to see that the protein levels correlate with the severity of the antisense plant phenotype.

Does Heme Regulate the ALA Synthesis at the Level of GluTR?

Based on the biochemical analysis it was postulated that plants contain two pools of ALA with distinct regulation, phytochrome or feedback regulated (Huang et al., 1990). In all plants studied so far, expression of at least one of the *HEMA* and *GSA* genes is known to respond to light, whereas the other members of these gene families are not responsive to light (Kumar et al., 1996b). It is not clear if expression of this set of *HEMA* and *GSA* genes is regulated by a feedback mechanism. The end product, heme, exhibited such a feedback inhibition on ALA biosynthesis (Beale, 1978). Lowering heme levels by iron chelators increased ALA accumulation (Duggan and Gassman, 1974). Of the two enzymes of the C_5 pathway, we believe that heme may not have a regulatory role on GSA-AT protein as our results in the present study show that decreased content of non-covalently bound hemes failed elevate the GSA-AT levels (Figs. 5 and 6). While this observation suggests that GluTR probably is the site of heme action, there is no conclusive evidence except an in vitro study in which GluTR activity was shown to be affected by heme (Huang and Wang, 1986). A direct proof would be the examination of the GluTR levels in antisense *GSA* transgenics or in mutant plants that synthesize suboptimal levels of heme.

Chlorophyll Deficiency in Antisense *HEMA1* Transgenics Is Not Due to Lowered Protochlorophyllide Oxidoreductase Levels

NADPH-dependent protochlorophyllide oxidoreductase catalyzes the reduction of protochlorophyllide to chlorophyllide in a light-dependent manner. Similar to *HEMA* and *GSA* genes, a set of *POR* genes (*PORA* and *PORB*) is involved in chlorophyll biosynthesis (Armstrong et al., 1995; Holtorf et al., 1995). Transfer of light-grown plants to the dark causes accumulation of Pchlide and decline of ALA synthesis. In light, however, Pchlide is reduced and ALA synthesis increases. The inverse levels of ALA and Pchlide in light and dark suggested the possible existence of a regulatory circuit between ALA and Pchlide synthesis (Beale, 1978). In the present study we observed that the anti-Por antibodies recognized a 36-kD protein in all the Arabidopsis lines including the control plants. While *PORA* mRNA disappears within a few hours of illumination (Armstrong et al., 1995), the expression of *PORB* proceeds throughout the greening process; thus, the detected signal was assumed to be the PorB protein. The constant levels of PorB suggested that the chlorophyll deficiency in the transgenic plants was primarily due to insufficient levels of Pchlide that had resulted from decreased ALA synthesis.

Is the C_5-Pathway the Sole Source for ALA in Plants?

While the existence of the C_5 pathway is widely accepted, other pathways of ALA formation in plants have been reported (Ramaswamy and Nair, 1973; Meller and Gassman, 1982). Efforts to thoroughly characterize these "alternative ALA-forming pathways" have been unsuccessful. More convincing yet indirect evidence for an extraplastidic ALA synthesis was obtained with a barley *albina* mutant (Hess et al., 1991, 1992). The lack of detectable levels of chloroplastic $tRNA^{Glu}$, and the presence of minute amounts of Pchlide, chlorophyll, and heme in these mutants indicated an additional pathway for tetrapyrrole biosynthesis.

We show that the levels of protoheme are decreased in the transgenic plants expressing antisense HEMA mRNA. On the other hand, a decrease (more than 50%) in absorption units at 588 nm was noticed in the severe phenotype (compared with the control); however, heme *a* was not detected as no heme *a* specific peaks were noticed in the absorption spectra. Nevertheless, radiotracer studies conducted with maize seedlings demonstrated that all of the cellular hemes including protoheme and the heme *a* (the mitochondrial heme, a marker for alternative pathway for tetrapyrrole biosynthesis) were made from ALA that is derived from C_5 pathway (Schneegurt and Beale, 1986). Furthermore, lethal effect produced in some lines by antisense *HEMA1* (present study) and antisense *GSA* (Höfgen et al., 1994) favors the C_5 pathway as a sole source for ALA biosynthesis.

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