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Differential Expression of Two *hemA* mRNAs Encoding Glutamyl-tRNA Reductase Proteins in Greening Cucumber Seedlings

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The first committed step of porphyrin synthesis in higher plants is the reduction of glutamyl-tRNA to glutamate 1-semialdehyde. This reaction is catalyzed by glutamyl-tRNA reductase, which is encoded by *hemA* genes. Two *hemA* cDNA clones (*hemA1* and *hemA2*) were obtained from cucumber (*Cucumis sativus*) cotyledons by the PCR and cDNA library screening. They showed significant homology with published *hemA* sequences. Southern blot analysis of cucumber genomic DNA revealed that these genes are located at different loci and that there is another gene similar to the *hemA* genes. Accumulation of *hemA1* mRNA was detected primarily in cotyledons and hypocotyls of greening cucumber seedlings, whereas that of *hemA2* mRNA was detected in all tissues examined. Illumination of cucumber seedlings increased markedly the accumulation of *hemA1* mRNA, but it did not induce remarkable changes in that of *hemA2* mRNA. These findings suggest that *hemA1* mRNA was accumulated in response to the demand of Chl synthesis in photosynthesizing tissues, whereas *hemA2* mRNA was expressed in response to the demand of the synthesis of porphyrins other than chlorophylls.

Chls and heme are major tetrapyrrole compounds in higher plants, playing essential roles in such processes as photosynthesis and respiration. These compounds are closed-macrocycle tetrapyrroles chelating Mg^{2+} (Chls) or Fe^{2+} (heme). In higher plants, they are synthesized exclusively from glutamyl-$tRNA^{Glu}$ via ALA, a linear five-carbon molecule (Beale and Weinstein, 1990). The branch point of the biosynthetic pathways of heme and Chls is protoporphyrin IX, a closed-macrocycle without chelated ions. Most reactions from glutamyl-$tRNA^{Glu}$ to protoporphyrin IX are believed to occur in the plastid (Smith, 1988; Beale and Weinstein, 1990).

In spite of common precursors, Chls and heme are subjected to different controls: heme are found in all cells, whereas Chls are found only in photosynthesizing tissues. Chls do not accumulate in dark-grown tissues in angiosperms, but a considerable amount of heme is accumulated (Ushimaru et al., 1992). Synthesis of Chl is greatly enhanced by light (Yoshida, 1991; Yoshida et al., 1995), but that of heme in chloroplasts is only slightly increased upon illumination (Ohashi et al., 1992).

During chloroplast development in higher plants, ALA formation is supposed to be a limiting step in Chl biosynthesis because (a) administration of exogenous ALA causes accumulation of porphyrin and Mg-porphyrin intermediates and (b) the rate of ALA formation parallels that of Chl accumulation (Beale and Weinstein, 1990). Heme biosynthesis is regulated at the steps of ALA formation, as in animals (Andrew et al., 1990), since accumulation of most of the later intermediates in the presence of light and molecular oxygen is toxic to the cell (Nordmann and Deybach, 1990).

ALA destined for all cellular tetrapyrroles is formed by the two-step conversion from glutamyl-tRNA; glutamyl-tRNA is first reduced to GSA in an NADPH-dependent reaction by GTR, and then GSA is transaminated by GSA aminotransferase to form ALA (Kannangara et al., 1988). Among these steps, reduction of glutamyl-tRNA is likely to be a regulatory point for the synthesis of heme and Chls because (a) it is the first step unique to ALA formation and (b) GTR is subjected to feedback regulation by heme (Huang and Wang, 1986; Beale and Weinstein, 1990; Pontoppidan and Kannangara, 1994). Although the existence of two routes for ALA synthesis was proposed by Huang and Castelfranco (1990), it has not been elucidated whether a single GTR protein or multiple GTR proteins are involved in the regulation of porphyrin synthesis in higher plants.

GTR proteins have been demonstrated to be encoded by *hemA* genes. Among higher plants, one *hemA* cDNA sequence has been reported with *Arabidopsis thaliana* (Ilag et al., 1994). We isolated two *hemA* cDNA clones from cucumber (*Cucumis sativus*) cotyledons to examine the two possibilities described above. One *hemA* gene was expressed in photosynthesizing tissues and was induced by illumination in greening cucumber seedlings; the other gene was expressed in every tissue examined, and its expression did not show a remarkable increase. These findings suggest

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Abbreviations: ALA, 5-aminolevulinic acid; GSA, glutamate-1-semialdehyde; GTR, glutamyl-tRNA reductase; WWW, world wide web.

that the expression of a *hemA* gene family regulates ALA synthesis.

MATERIALS AND METHODS

Plant Materials

Seeds of cucumber (*Cucumis sativus* L. cv Aonagajibai, purchased from Takii Seeds Co., Kyoto, Japan) were soaked in tap water for several hours and germinated on moist vermiculite in darkness at 28°C. For the synthesis of cDNA, seedlings were grown for 4 d and exposed to white light (Toshiba [Tokyo, Japan] FLR80W/A white fluorescent lamps) at an intensity of approximately 18.9 W/m^2. For Southern blot analysis, seedlings were grown for 5 d in the light. For northern blot analysis, seedlings were grown for 5 d and exposed to white light as described above or kept in darkness as a control.

Bacterial Strains

Escherichia coli strain DH5α was used for cloning into plasmids. *E. coli* strains C600Hfl and Y1088 were used for work with the λgt10 and λgt11 phages. A *hemA*-deficient mutant of *E. coli*, LE392Δ*hemA* (Nakayashiki et al., 1995), was used for functional complementation analysis.

PCR Amplification of *hemA* cDNA Fragments

Total RNA was extracted from 10 g fresh weight of cotyledons by the phenol/SDS method, and poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose according to Ausubel et al. (1987). cDNA was prepared by first-strand synthesis with murine reverse transcriptase (First-Strand Synthesis kit, Pharmacia). The consensus amino acid sequence of published *hemA* sequences and a barley *hemA* cDNA clone isolated by B. Grimm (personal communication) were used to design the following oligonucleotides: I, 5′-GAATTCACIGCICCIGTIGA(TC)-ATG(AC)G(GATC)GAA-3′; II, 5′-GAATTCACITG(TC)-AA(TC)(AC)GIATGGA(GA)AT(GATAC)TAT-3′; and III, 5′-GAATTCAC(CT)TGICCIA(AG)IAT(TC)TGICC(TC)TC-(GATC)CC-3′. Oligonucleotides I, II, and III were designed from regions of the predicted amino acid sequence of the barley cDNA clone: I, ThrAlaProValAspMetArgGlu; II, ThrCysAsnArgMetGluIleTyr; III, GlyGluGlyGlnIleLeuAla-GlnVal. Using the constructed cDNA as a template, oligonucleotide I or II as a forward primer, and oligonucleotide III as a reverse primer, DNA sequences were amplified by PCR (30 cycles of denaturing, annealing, and polymerization were carried out at 94°C for 1 min, 48°C for 1 min, 72°C for 3 min, respectively). A part of *hemA1* cDNA was amplified with primers I and III and parts of both *hemA1* and *hemA2* cDNA were amplified with primers II and III. PCR products were run on low-melting-point agarose and extracted from the gel. Purified DNA fragments were ligated to pUC119 and used to transform *E. coli* DH5α. Inserts were sequenced as described below and found to be *hemA* cDNA fragments.

Construction and Screening of cDNA Libraries

Poly(A)$^+$ RNA isolated as described above was used to synthesize double-stranded cDNA with a TimeSaver cDNA synthesis kit (Pharmacia) according to the manufacturer's specifications. cDNA was cloned into λgt10 and λgt11 phage vectors using Gigapack gold packaging extracts (Stratagene).

The libraries were screened using standard procedures (Sambrook et al., 1989) with the PCR fragments described above, which were ^{32}P-labeled with a BcaBest labeling kit (Takara Shuzo Co., Shiga, Japan). Positive clones were subsequently purified, and the *Not*I inserts of clones were recloned into the pBluescript II plasmid vector (Stratagene).

Sequencing and Sequence Analysis

Nucleotide sequencing by the dideoxy chain-termination method (Sanger et al., 1977) was performed using a Sequenase 2.0 kit (United States Biochemical). Sequence analysis was performed using the computer software Genetyx-Mac (Software Development, Tokyo, Japan). Multiple alignment of the amino acid sequences and phylogenic analysis were done using the computer software Clustal W (Thompson et al., 1994) with the default values. Protein-sorting analysis was done using the computer server PSORT (Nakai and Kanehisa, 1992) at the National Institute for Basic Biology (Okazaki, Japan), on a WWW site on the Internet, http://psort.nibb.ac.jp/. Homology search was done at the National Institute of Agrobiological Resources (Tsukuba, Japan) DNA Information Stock Center, on a WWW site on the Internet, http://www.dna.affrc.go.jp/.

Functional Complementation Analysis

The parts of *hemA* cDNA clones that we predicted to encode mature proteins were amplified by PCR (see "Results"). Amplified fragments were ligated with the pGEX-2T (Pharmacia) plasmid vector. The resulting plasmids were introduced into the LE392Δ*hemA*-competent cells prepared as described by Inoue et al. (1990). Transformants were plated on Luria broth plates containing 1% trypton, 0.5% yeast extract, 1% NaCl, and 50 μg/mL ampicillin. Complemented cells produced normal-sized colonies, and other cells produced dwarf colonies on the plates.

Southern Analysis

Genomic DNA was prepared from cucumber cotyledons according to the method of Ausubel (1987). DNA (6 μg) was digested with *Apa*I, *Bam*HI, *Eco*RI, and *Xba*I. Restriction fragments were subjected to electrophoresis in a 0.5% (w/v) agarose gel and then transferred to Biodyne-B membrane (Pall, East Hills, NY) by the alkaline transfer method according to the manufacturer's specifications. The resulting blot was hybridized with radiolabeled probes and prepared as described above for library screening in 20% formamide, 5× SSC, 5× Denhardt's reagent, 0.5% SDS, and 100 μg mL^{-1} denatured salmon sperm DNA at 42°C. The blot was washed three times in 5× SSC, 0.1% SDS, twice in

2× SSC, 0.1% SDS, twice in 1× SSC, 0.1% SDS, and twice in 0.2× SSC, 0.1% SDS at 42°C. After the washing buffer was changed, the blot was exposed on an Imaging Plate (Fujifilm, Tokyo, Japan) and analyzed with a radioimage analyzer, BAS1500 (Fujifilm).

Northern Analysis

Total RNA was extracted from cucumber cotyledons, hypocotyls, and roots as described by Yoshida et al. (1995). Total RNA (4 μg) was electrophoresed in a 1% agarose/formamide gel and transferred to Biodyne-B membrane (Pall). The blot was irradiated with UV light (UV Stratalinker 2400, Stratagene) and then hybridized in 50% formamide, 5× SSPE, 5× Denhardt's reagent, 0.5% SDS, 100 μg mL^{-1} denatured salmon sperm DNA at 42°C with radiolabeled probes. The blot was washed three times in 5× SSC, 0.1% SDS, twice in 2× SSC, 0.1% SDS, twice in 1× SSC, 0.1% SDS, and twice in 0.2× SSC, 0.1% at 42°C. The blot was exposed on an Imaging Plate (Fujifilm), and analyzed with a radioimage analyzer, BAS1500 (Fujifilm). A PCR-amplified fragment of α-tubulin cDNA of *A. thaliana* was used as a control.

RESULTS

Isolating Two *hemA* cDNA Clones from Cucumber Cotyledons

Using two pairs of primers that were designed according to the conserved amino acid sequences in the middle parts of GTR proteins of *Hordeum vulgare* (B. Grimm, personal communication), *Synechocystis* (Verkamp et al., 1992), *E. coli* (Verkamp and Chelm, 1989), *Bacillus subtilis* (Petricek et al., 1990), *Chlorobium vibrioforme* (Majumdar et al., 1991), we carried out the PCR with a single-stranded cDNA pool prepared from cucumber cotyledons. We obtained two amplified cDNA species and named them *hemA1* and *hemA2*.

We used the amplified *hemA1* fragment as a probe to isolate a full-length cDNA clone from a λgt11 library of greening cucumber cotyledons. We obtained 10 clones after tertiary screening of 5×10^5 primary plaques. One of these clones was recloned into a pBluescript II plasmid vector and further analyzed.

A cloned PCR product of *hemA2* was used as a probe to isolate a full-length clone of *hemA2* from a λgt10 cDNA library produced from the same cDNA pool as the λgt11 cDNA library. Ten plaques remained positive after tertiary screening of 5×10^5 primary plaques. As judged by restriction enzyme digestion patterns, two of them were clones of the *hemA2* cDNA species and the others were clones of the *hemA1* species. The *hemA2* probe we used in the screening weakly hybridized to the *hemA1* cDNA clones in our screening conditions. One of the *hemA2* clones was recloned into pBluescript II plasmid vector and further analyzed.

Sequence Analysis of the Two *hemA* cDNA Clones

Figure 1 shows the cDNA sequences and the derived amino acid sequences of *hemA1* and *hemA2*. The cDNA sequence of *hemA1* contains one open reading frame starting from nucleotide 78 with an ATG start codon and ending at nucleotide 1736 with a TAA stop codon. The open reading frame encodes a polypeptide chain of 552 amino acid residues with a calculated molecular mass of 61 kD.

The cDNA sequence of *hemA2* contains one open reading frame starting from nucleotide 91 with an ATG start codon and ending at nucleotide 1736 with a TAG stop codon. The open reading frame encodes a polypeptide chain of 542 amino acid residues with a calculated molecular mass of 60 kD.

The deduced amino acid sequence of the *hemA1* clone is 77.1% identical to that of the *hemA2* clone and from 29.3% (*C. vibrioforme*) to 76.1% (*A. thaliana*) identical to those of other organisms listed in Figure 2. The deduced amino acid sequence of the *hemA2* clone is from 30.4% (*C. vibrioforme*) to 72.3% (*A. thaliana*) identical to those of other organisms listed in Figure 2. Conserved amino acid residues are found through the entire sequences, especially the regions between positions 151 and 169, 200 and 233, and 252 and 284, which are highly conserved (Fig. 2; the position numbers are of the corresponding amino acids in the sequence of *hemA1*.). The region between 200 and 233 is involved in the GTR activity: the substitution of Cys residue for Tyr at position 206 in *B. subtilis* (Petricek et al., 1990) resulted in the ALA auxotroph phenotype, and the substitution of Ser residue for aspartate at the same position in *E. coli* resulted in a weak *hemA*$^-$ phenotype (Nakayashiki et al., 1995). The region between positions 289 and 321 is predicted to form a $\beta\alpha\beta$ structure for ADP binding (Wierenga et al., 1986; Petricek et al., 1990; Ilag et al., 1994) and is similar to amino acid sequences of NADH- or NADPH-binding proteins such as *Streptococcus thermophilus* UDP-Glc 4-epimerase (SwissProt accession No. A44509) and *Streptomyces fradiae* varine dehydrogenase (SwissProt accession No. P40176).

The amino acid sequences of higher plants have long N-terminal extensions among which there is a lower similarity. This region is predicted to be a chloroplast transit peptide, since a GTR protein was purified from the stroma of barley chloroplasts (Pontoppidan and Kannangara, 1994). This prediction was supported by computer calculations using a WWW server, PSORT, which predicts protein sorting signals and localization sites in amino acid sequences (Nakai and Kanehisa, 1992). It predicted that the *hemA1* product would localize in the chloroplast stroma with a certainty of 0.950 and in the mitochondrial matrix space with a certainty of 0.547, and that the *hemA2* product would localize in the chloroplast stroma and in the mitochondrial matrix space with certainties of 0.520 and 0.360, respectively.

Konishi et al. (1993) proposed that the amino acid sequences around the transit peptides have a common secondary structure, β-turn/β-sheet/α-helix. We predicted the secondary structures of the N-terminal sequences of the *hemA1* and *hemA2* products by the method of Chou and Fasman (1978) and found the region that matched the common structure (data not shown). We predicted that the cleavage site of the *hemA1* product is between positions 79 and 80, before the Ser residue, and that the cleavage site of

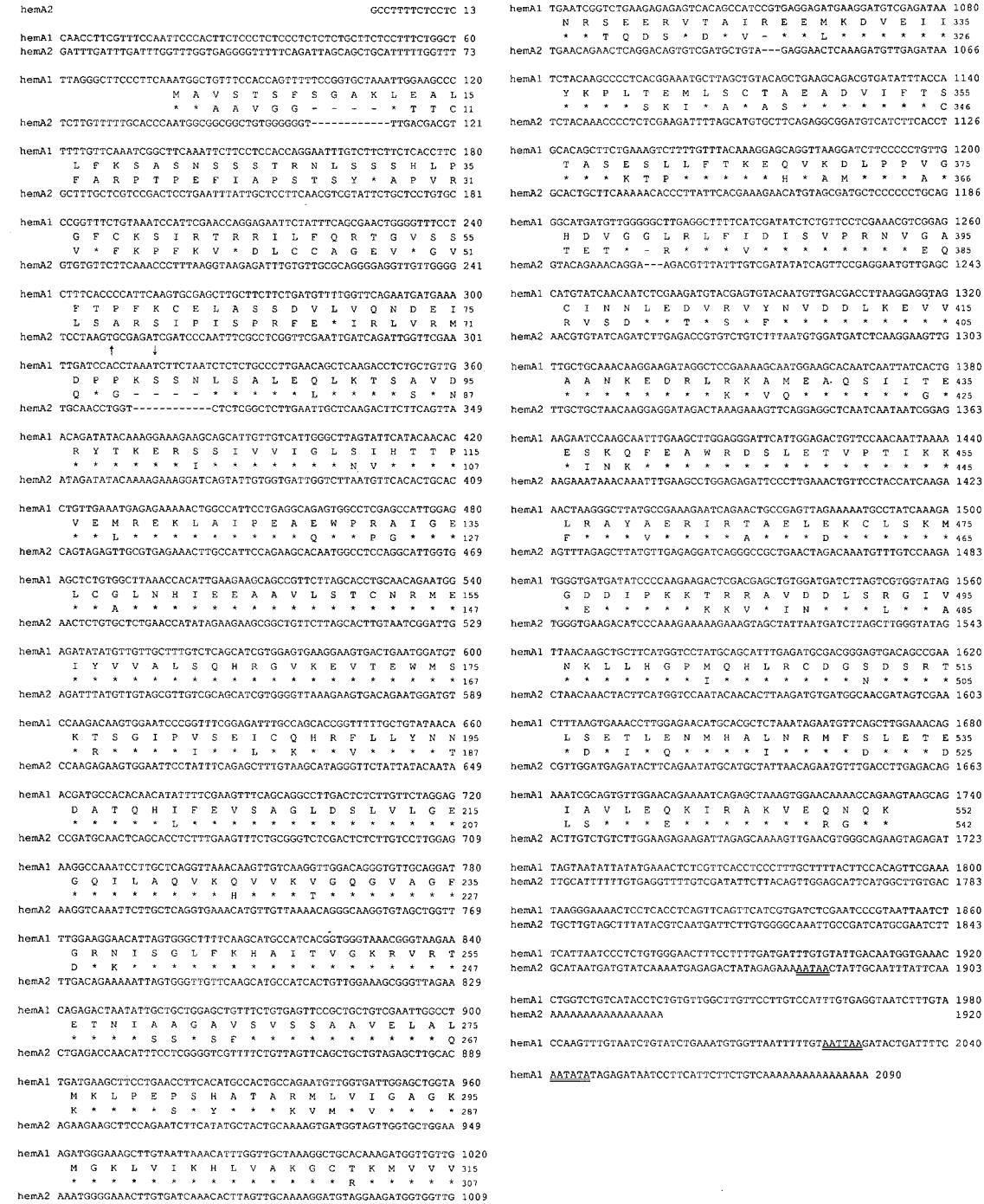

Figure 1. The nucleotide and deduced amino acid sequences of cDNA of *hemA1* and *hemA2* cDNA clones. The sequences are aligned by Clustal W (Thompson et al., 1994) so that the amino acid sequences are best aligned. The arrows show the predicted transit sequence cleavage sites. Putative polyadenylation signals are double-underlined.

the *hemA2* product is between positions 53 and 54, before the Ala residue (Fig. 1).

Functional Complementation of a *hemA*-Deficient Strain of *E. coli*

To check if these cDNA clones encoded functional GTR proteins, we introduced them into a *hemA*-deficient strain of *E. coli*, LE392Δ*hemA*, which lacked the middle half of the *hemA* gene and showed an ALA auxotroph phenotype (Nakayashiki et al., 1995). The full-length clones of *hemA1* and *hemA2* did not complement the strain. Since the clone of *hemA1* contained a predicted untranslated region and a predicted transit peptide for the chloroplast (see above), we removed most of this region (1–210 bp) and introduced it in

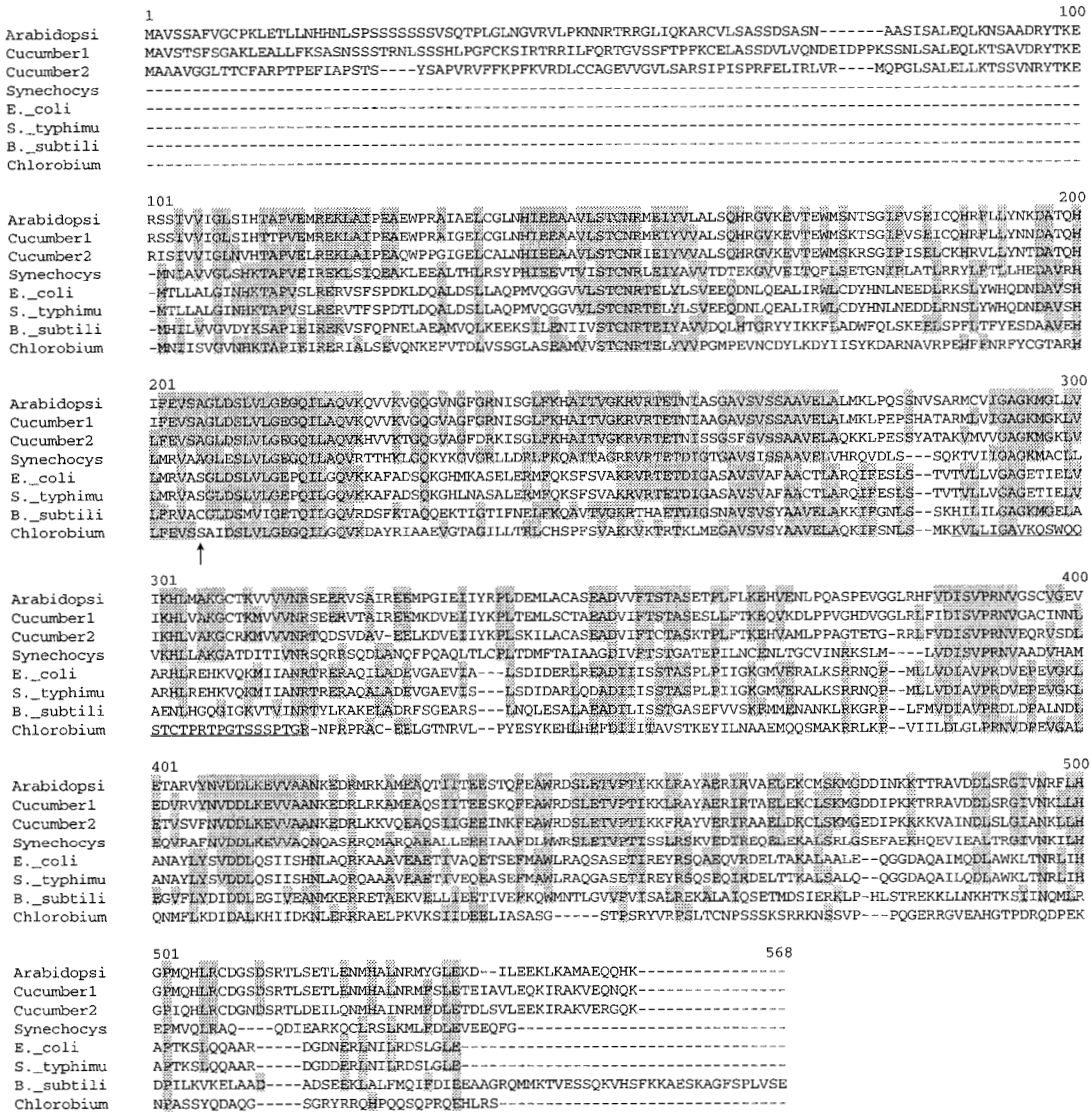

Figure 2. Alignment of the amino acid sequences of various GTR proteins. The predicted amino acid sequences of the cucumber GTR proteins (Cucumber1 and Cucumber2) was aligned with the those of the GTR proteins from *A. thaliana* (Arabidops; Ilag et al., 1994), *Synechocystis* (Synechocys; Verkamp et al., 1992), *E. coli* (E. coli; Verkamp and Chelm, 1989), *Salmonella typhimurium* (S. typhimu; Elliot, 1989), *B. subtilis* (B. subtili; Petricek et al., 1990), and *C. vibrioforme* (Chlorobium; Majumdar et al., 1991) by introducing gaps to maximize identity using the computer software Clustal W with the default values (Thompson et al., 1994). Residues shared by more than four of the sequences are shaded. The numbers refer to the positions of the corresponding amino acids in the sequence of *hemA1*. An arrow indicates the residue playing an essential role in the GTR activity (Petricek et al., 1990; Nakayashiki et al., 1995). The region that is predicted to fold into a $\beta\alpha\beta$ unit with ADP-binding properties (Wierenga et al., 1986; Petricek et al., 1990; Ilag et al., 1994) is underlined.

pUC119 into LE392Δ*hemA*. The ALA auxotroph was supplemented, suggesting that the *hemA1* clone encoded a functional GTR protein (data not shown). We removed the predicted 5′ untranslated region (1–90 bp) and the predicted transit peptide (91–249 bp) from the *hemA2* cDNA clone and ligated it with a pGEX-2T plasmid vector. The resulting clone did not complement the *E. coli* strain (data not shown).

Southern Analysis of Cucumber Genomic DNA

To estimate the copy number of *hemA* genes in cucumber, we performed Southern blot analysis. Cucumber DNA was digested with four restriction enzymes and hybridized with labeled DNA probes of the fragment from 525 to 742 bp of *hemA1* cDNA or the fragments that extended from 514 to 731 bp of *hemA2* cDNA. These fragments contained highly conserved regions of the *hemA* genes and were expected to hybridize to other *hemA*-like genes. The *hemA1* probe strongly hybridized with a single fragment under high-stringency rinse conditions (0.1× SSC, 0.5% SDS, 42°C) (Fig. 3, left). Under the same conditions, the *hemA2* probe strongly hybridized with another single band (Fig. 3, middle). Both probes weakly hybridized with the bands that corresponded to each other. Upon lower stringency of

rinsing (1× SSC, 0.5% SDS, 42°C), the *hemA2* probe hybridized with an additional restriction fragment other than *hemA1* and *hemA2* (Fig. 3, right), suggesting that there was another *hemA* gene in the cucumber genome. At present, we cannot confirm the expression of this gene.

Differential Expression of *hemA1* and *hemA2* Genes among Tissues in Cucumber Seedlings

Most of the Chls were found in cotyledons; a small amount of them was found in hypocotyls and no Chls accumulated in roots. To study the relationship between the activity of Chl synthesis and expression of *hemA* genes, we examined the steady-state levels of *hemA* mRNAs among different tissues in cucumber seedlings. Total RNA was extracted from cotyledons, hypocotyls, or roots of the seedlings that were grown for 5 d in darkness and illuminated with white light for 24 h. It was blotted and analyzed by northern hybridization using labeled DNA probes of the predicted 3′ untranslated regions of *hemA1* and *hemA2* cDNAs. *hemA1* mRNA was detected in cotyledons and hypocotyls (Fig. 4A). *hemA1* mRNA seemed to be accumulated only in photosynthesizing tissues. *hemA2* mRNA was

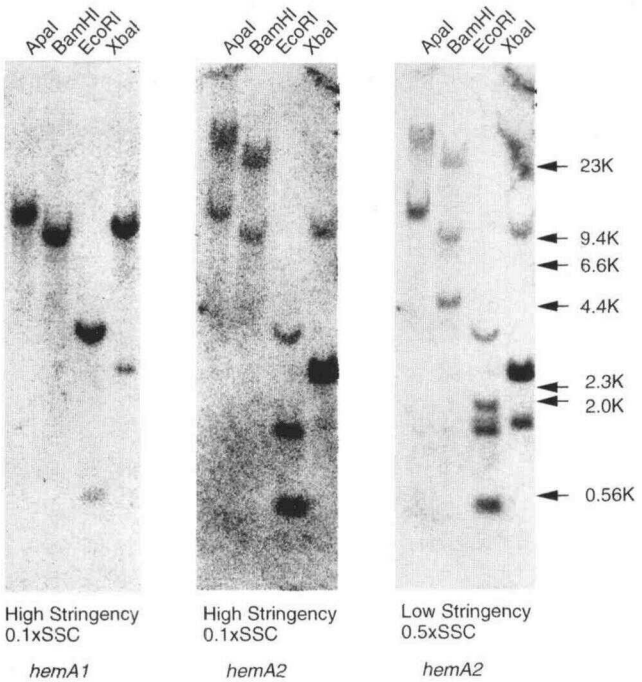

Figure 3. Genomic Southern analysis of *hemA* genes in cucumber. Total genomic DNA (4.8 μg) was digested with *Apa*I, *Bam*HI, *Eco*RI, and *Xba*I. The DNA samples were then separated on a 0.5% agarose gel and blotted onto nylon membranes. Left, Blot hybridized with the *hemA1* homologous probe consisting of the region spanning nucleotides 525 to 742 and washed under high-stringency conditions (0.1× SSC, 0.5% SDS, 42°C). Middle, Blot hybridized with the *hemA2* homologous probe consisting of the region spanning nucleotides 514 to 731 and washed under high-stringency conditions (0.1× SSC, 0.5% SDS, 42°C). Right, Blot hybridized with the *hemA2* homologous probe consisting of the region spanning nucleotides 514 to 731 and washed under low-stringency conditions (0.5× SSC, 0.5% SDS, 42°C).

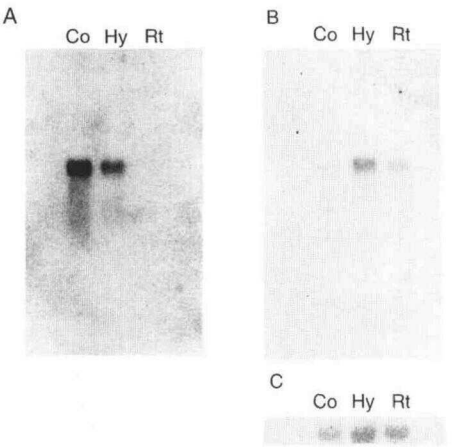

Figure 4. Northern blot analysis of total RNA of various tissues of cucumber seedlings. Cucumber seedlings were grown for 5 d in darkness and illuminated for 24 h. Total RNA was isolated from cotyledons, hypocotyls, and roots of the seedlings. A, An RNA blot containing total RNA (4 μg) isolated from the tissues was hybridized with the specific probe of the *hemA1* cDNA clone consisting of the region spanning nucleotides 1621 to 1961. B, An RNA blot containing total RNA (4 μg) isolated from various tissues was hybridized with the specific probe of the *hemA2* cDNA clone consisting of the region spanning nucleotides 1604 to 1883. C, An RNA blot containing total RNA (4 μg) isolated from various tissues was hybridized with the *A. thalinana* α-tubulin gene. Co, Cotyledons; Hy, hypocotyls; Rt, roots.

detected primarily in hypocotyls and roots (Fig. 4B). A small amount of *hemA2* mRNA was detected in cotyledons (Figs. 4B and 5B). *hemA2* mRNA was detected in all tissues examined.

Rapid Induction of *hemA1* mRNA Accumulation by White Light

When etiolated seedlings are exposed to light, Chl rapidly accumulates in cotyledons after a 2-h lag phase. Since the reduction of glutamyl-tRNA is considered to be a regulation site in porphyrin synthesis, we examined the effect of light on the steady-state levels of *hemA* mRNAs. Total RNA was extracted from 5-d-old etiolated cotyledons and hypocotyls that were illuminated for various periods and was analyzed by northern hybridization using the probes of the predicted 3′ untranslated region of *hemA1* and *hemA2* cDNAs. Expression of the *hemA1* gene was low in the dark and during the lag phase for Chl synthesis in cotyledons (Fig. 5A). After 3 h of illumination, the levels of *hemA1* mRNA were drastically increased. Hypocotyls exhibited the same profile of *hemA1* mRNA accumulation as cotyledons (Fig. 5C). A small amount of *hemA2* mRNA was detected in cotyledons and it increased slightly with a peak at 12 h of illumination (Fig. 5B). In hypocotyls, a considerable amount of *hemA2* mRNA accumulated in the dark and decreased after onset of illumination (Fig. 5D).

DISCUSSION

The predicted amino acid sequences of both clones have high similarities with published sequences of GTR proteins.

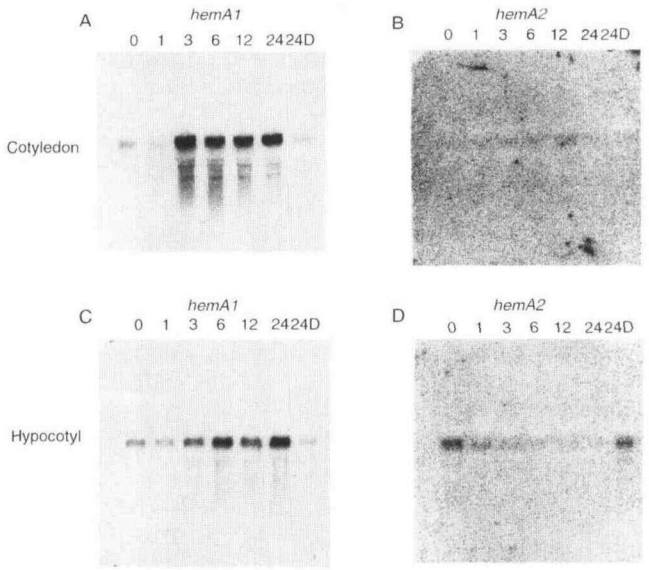

Figure 5. Northern blot analysis of total RNA from cucumber cotyledons greening under white light. Cucumber seedlings were grown for 5 d and exposed to white light for the number of hours indicated above each lane or kept in darkness as a control (lanes 24D). Total RNA was isolated from cotyledons and hypocotyls of the seedlings. Four micrograms of total RNA per gel lane were electrophoresed and blotted onto nylon membranes. A, An RNA blot containing total RNA from cotyledons was hybridized with the specific probe of *hemA1*. B, An RNA blot containing total RNA from cotyledons was hybridized with the specific probe of *hemA2*. C, An RNA blot containing total RNA from hypocotyls was hybridized with the specific probe of *hemA1*. D, An RNA blot containing total RNA from hypocotyls was hybridized with the specific probe of *hemA2*.

Some regions of the GTR amino acid sequences are well conserved (Fig. 2). One of the conserved regions, between positions 289 and 321, is predicted to be a $\beta\alpha\beta$ structure, which is involved in NAD binding (Wierenga et al., 1986). Also conserved is the region between positions 200 and 233, which was proposed to be essential in the enzyme activity (Petricek et al., 1990; Nakayashiki et al., 1995).

The predicted amino acid sequences of *hemA1* and *hemA2* mRNA have N-terminal extensions, which were predicted to be transit peptides for chloroplasts by PSORT, a WWW server for analyzing and predicting protein-sorting signals coded in amino acid sequences. This prediction is consistent with the observation that ALA in plants is synthesized in plastids from glutamyl-tRNA (Beale and Weinstein, 1990).

The cDNA clone of *hemA1* complemented a *hemA*-deficient mutant of *E. coli*, LE392Δ*hemA*, when parts of the 5′ sequence of the clones were removed. This demonstrated that the clone encoded a functional GTR protein. By contrast, the cDNA clone of *hemA2* did not complement LE392Δ*hemA* when the sequence was ligated into the pGEX-2T plasmid vector. There are several possible explanations for these results: (a) The translated polypeptide of the *hemA2* cDNA clone was not correctly folded. In some cases, a transit peptide inhibits a protein activity. Since we did not know the first amino acid residue of the mature protein, we could not construct a cDNA clone that encoded only the amino acid residues of the mature protein. (b) The protein did not accept the $tRNA^{Glu}$ of *E. coli*. *Chlorobium* GTR can reportedly discriminate between different tRNA molecules from different organisms (Avissar and Beale, 1990). (c) In *E. coli*, the activity of the *hemA2* cDNA product was inhibited more strongly than that of the *hemA1* product by some substances such as heme. Although the *hemA2* cDNA clone did not complement the *E. coli* mutant, we believe it encoded a functional GTR protein because it was expressed and its expression seemed to be controlled in cucumber seedlings.

hemA1 mRNA seems to be expressed in response to the need for Chl biosynthesis. Accumulation of *hemA1* mRNA was detected in Chl-synthesizing tissues, such as cotyledons and hypocotyls. It was enhanced by illumination and changed in a pattern similar to that of Chl synthesis (Fig. 5, A and C). Expression of *hemA1* mRNA might regulate the rate of Chl synthesis.

hemA2 mRNA seems to be expressed in response to the need for porphyrins other than Chls, e.g. heme. Accumulation of *hemA2* mRNA was detected primarily in hypocotyls and roots, which synthesize few if any Chls. A small amount of *hemA2* mRNA accumulation was detected in cotyledons. The existence of *hemA2* mRNA in cotyledons was also supported by the isolation of its cDNA clone from the cDNA library of the cotyledons. *hemA2* mRNA accumulation was not changed markedly in cotyledons upon illumination with white light and decreased in hypocotyls. *hemA2* mRNA accumulation was controlled independently of Chl formation. The need for porphyrin synthesis is possibly different among tissues, when seedlings are fed by light. It might be reflected in the different profiles of *hemA2* mRNA expression, which was observed in cotyledons and hypocotyls (Fig. 5, B and D). Although no feedback regulation by GSA or ALA was reported in plants, an excess amount of GSA or ALA made by the product of light-induced *hemA1* gene might repress the *hemA2* gene expression in hypocotyls.

A GTR protein was purified from the stroma of barley chloroplasts. GTR has been suggested to be a tetramer or a hexamer (Pontoppidan and Kannangara, 1994). Since *hemA1* and *hemA2* were differently expressed among tissues, it is unlikely that both the *hemA1* and *hemA2* products are heteromers.

Southern analysis of cucumber genomic DNA revealed that there is another *hemA*-like gene in addition to *hemA1* or *hemA2*. We do not know if this gene is expressed or not. Small *hemA* gene families were found by genomic Southern analysis in cucumber (this work), Arabidopsis (Ilag et al., 1994), and barley (B. Grimm, personal communication). Two different cDNA clones have been isolated from cucumber (this work), barley (B. Grimm, personal communication; R. Tanaka, unpublished data), and Arabidopsis (Ilag et al., 1994; M. Kumar, personal communication).

There are two genes encoding GSA aminotransferase in *A. thaliana* (Ilag et al., 1994) and soybean (Frustaci et al., 1995). Although there is no direct evidence for the existence

of two genes encoding GSA aminotransferase in cucumber, the existence of two routes for ALA synthesis has been suggested; one route was inhibited by gabaculine, a potent inhibitor of GSA aminotransferase, whereas the other was not (Huang and Castelfranco, 1990). Two GTR and two GSA aminotransferase proteins might constitute two routes of ALA synthesis in cucumber seedlings that seem to be regulated in response to the demand for Chls and other porphyrin compounds, respectively.

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