Two Types of Ferrochelatase in Photosynthetic and Nonphotosynthetic Tissues of Cucumber

THEIR DIFFERENCE IN PHYLOGENY, GENE EXPRESSION, AND LOCALIZATION*

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Ferrochelatase catalyzes the insertion of Fe\textsuperscript{2+} into protoporphyrin IX to generate protoheme. In higher plants, there is evidence for two isoforms of this enzyme that fulfill different roles. Here, we describe the isolation of a second ferrochelatase cDNA from cucumber (CsFeC2) that was less similar to a previously isolated isoform (CsFeC1) than it was to some ferrochelatases from other higher plants. In 

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sis of localization was prevented due to the lack of specific antibodies against plant ferrochelatases. Moreover, Nakayama et al. (18) showed that one subunit of Mg-ferrochelatase (ChlH) was reversibly dissociated from the envelope to the stroma at low Mg\(^{2+}\) concentrations and that the change in localization of ChlH may be physiologically important to regulate the amount of chlorophyll. Therefore, it is important to determine accurately the localization of the ferrochelatase protein for a better understanding of the strict coordination of heme and chlorophyll synthesis in chloroplasts.

We previously succeeded in the functional expression of cucumber ferrochelatase (HemH; CsFeC1 as designated in this report) cloned by Miyamoto et al. (14) and in producing antibodies against the enzyme protein. We showed that this ferrochelatase was localized to nonphotosynthetic tissues (19). However, in cucumber, ferrochelatase activity was detected in the chloroplasts of photosynthetic tissue, indicating the existence of another ferrochelatase(s) for heme biosynthesis in photosynthetic tissues under different control.

In this study, we describe the isolation of a plastidic ferrochelatase cDNA from cucumber and its intraplastidic location, membrane binding, formation of complexes, and mRNA accumulation. Analyses of location and mRNA accumulation of the two cucumber ferrochelatases suggest that there are two different biosynthetic pathways for tetrapyrroles in photosynthetic and nonphotosynthetic tissues, respectively.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—Etiolated cucumber seedlings (Cucumis sativus L. cv Aonagijiba) were grown on moist gauze at 26 °C in a growth chamber in darkness for 4 days and then subjected to various illumination routines and cycloheximide treatment.

**cDNA Cloning**—A cDNA library of RNA from cucumber cotyledons, grown in darkness for 4 days following illumination for 24 h, was made using the ZAP-cDNA Synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. PCR was carried out using several sets of degenerate oligonucleotide primers designed from the region conserved among ferrochelatases of plants with the cDNA library as the template. PCR amplification was carried out for 30 cycles (1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C) using Takara Ex Taq (Takara Shuzo, Otsu, Japan) according to the manufacturer’s instructions. The amplified product was ligated into pBluescript SK(−) plasmid and digested with EcoRI and XhoI. The obtained fragment was ligated into expression vector pGEX-2T (Amersham Biosciences, Inc.) predigested with EcoRI and XhoI. This plasmid was transformed into Escherichia coli XL1-Blue MRF.

**Purification of the Deduced Mature Region of CsFeC2—**E. coli transformants in which GST-CsFeC2 was induced by isopropyl-\(\beta\)-D-thiogalactopyranoside were harvested and resuspended in extraction buffer (50 mM Tris-HCl, pH 7.7, 20% glycerol, and 1 mM dithiothreitol). The cells were disrupted by sonication twice for 10 min each time. To this lysate, 10° Triton X-100 added to 0.5% to solubilize the fusion protein, and the lysate was incubated for 30 min. The lysate was then centrifuged at 10,000 × g for 5 min at 4 °C, and 50% slurry of glutathione-Sepharose 4B (Amersham Biosciences, Inc.) was added to the supernatant. The supernatant was precipitated by centrifugation at 500 × g for 5 min in SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 18% sucrose, 10% (v/v) β-mercaptoethanol, and 4% SDS) and subjected to SDS-PAGE. The GST-CsFeC2 fusion protein was eluted by homogenizing the sliced gel with extraction buffer. The extracted protein was digested with thrombin and separated by SDS-PAGE after boiling. CsFeC2 was extracted from the gel slice by homogenization in the presence of 0.1% SDS. The purified CsFeC2 was used to determine its N-terminal amino acid sequence and for immunization.

**Antibody Preparation and Purification**—A rabbit was immunized with ~300 μg of purified CsFeC2 with complete or incomplete adjuvant (Nacalai Tesque, Kyoto, Japan). The obtained antisera was purified by the method of epitope selection (21) and used for Western blotting analyses.

**Western Blotting Analysis**—Proteins of the various extracts were separated on SDS-PAGE gels and then electroblotted onto nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with the CsFeC2 antibodies and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (anti-rabbit IgG (H+L); Vector Laboratories, Burlingame, CA). The bands of cucumber ferrochelatase were detected using ECL Western blotting detection reagents (Amersham Biosciences, Inc.).

**Subcellular Fractionation**—Cucumber chloroplasts and mitochondria were isolated from the cotyledons of seedlings illuminated for 40 h. Chloroplasts and their fractions (thylakoid, envelope, and stroma) were prepared by the method described by Douce and Joyard (22). Intact chloroplasts were recovered at the interface of 40/80% Percoll at 6,000 × g for 15 min at 4 °C. Subfractions of chloroplasts were purified by discontinuous sucrose density gradients from lysed purified chloroplasts. The stroma was recovered in the upper layer at 72,000 × g for 1 h at 4 °C, the thylakoids were in the pellet under the 0.93 M sucrose layer, and the envelope was between 0.6 and 0.93 M sucrose layer. The fractions of thylakoid and envelope membranes were washed several times.

Mitochondria were isolated essentially as described by Nishimura et al. (23), with slight modifications. The homogenate of cucumber cotyledons was centrifuged at 2,000 × g for 10 min, and the supernatant was centrifuged at 10,000 × g for 10 min. The pellet was resuspended in a solution of 20 mM HEPES-KOH, pH 7.2, 0.3 mM mannitol, and 0.1% bovine serum albumin. A discontinuous Percoll gradient was prepared by layering 1.2 ml of 60% and 2 ml each of 45%, 25%, and 5% Percoll solutions. Then 1 ml of the suspension was layered on top of this gradient and centrifuged at 30,000 × g for 30 min at 4 °C (RPS40T swing rotor; Hitachi). The mitochondrial fraction was recovered at the interface of the 45% and 25% layers and washed under the same conditions as described for the discontinuous Percoll gradient. To check the mitochondrial viability of freshly isolated mitochondria, a swelling assay was performed as follows. The mitochondrial fraction and 0.05 mM sodium phosphate buffer (pH 7.3) containing 0.05 mM malate were incubated at 25 °C, and the amount of the reaction product, fumarate, was determined by measuring the absorbance at 250 nm.

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3 D. P. Singh and A. G. Smith, unpublished data.
were solubilized at 4°C (containing 10%/H9262 glycerol, and SDS (0.4%, 1%, or 2%). Aliquots of 20°C ferrochelatases, respectively. shaded for prokaryotic and eukaryotic

alignment of C-terminal amino acid sequences of CsFeC2, AtFC-II, rice ferrochelatase (OsFeC), and Synechocystis PCC 6803 ferrochelatase (6803 FeC) with the third membrane-spanning helix of early light-inducible protein of soybean (GmELIP) and Arabidopsis (AtELIP), small Cab-like proteins of Synechocystis PCC 6803 (ss1683, ssr2559, ssr2542, and ssr1789), HLIP of Synechococcus PCC 7942 (7942 HLIP), and the third membrane-spanning helix of Cab proteins of Arabidopsis (Lhca2, Lhca5, Lhcb2, Lhcb3, and Lhcb5). Stars indicate the amino acids conserved among all aligned proteins.

Electrophoresis of Partially Denatured Thylakoid—Chlorophyll-protein complexes were separated as described by Andersson et al. (24), with slight modifications. Thylakoids equivalent to 50 μg of chlorophyll were solubilized at 4°C in 100 μl of 0.3 M Tris-HCl, pH 8.8, 13% glycerol, and SDS (0.4%, 1%, or 2%). Aliquots of 20 μl of this solution (containing 10 μg of chlorophyll) were subjected to electrophoresis in an 8–11% polyacrylamide gradient gel containing 0.1% SDS. The upper reservoir buffer (pH 8.64) was 41 mM Tris borate/0.1% SDS, and the lower reservoir buffer was 0.43 M Tris borate/0.1% SDS, and the third membrane-spanning helix of Cab proteins of Arabidopsis (Lhca2, Lhca5, Lhcb2, Lhcb3, and Lhcb5).

Northern Blotting Analyses—Aliquots of 20 μg of total cucumber RNA were used for Northern blotting. A conserved region (nucleotides 398–1848) of CsFeC2 cDNA was used as a probe, followed by a fragment of Arabidopsis 25 S rRNA cDNA used as a control for the amount of RNA in each track.

Treatment with Cycloheximide—The cotyledons excised from 4-day-old etiolated seedlings were incubated in darkness on filter paper moistened with 3.5 ml of cycloheximide (100 μM) in Petri dishes.

RESULTS

Cloning of CsFeC2 cDNA—Primers for degenerate PCR were constructed from the amino acid sequences highly conserved among ferrochelatases of plants. PCR amplification using a cDNA library of cucumber cotyledons as a template produced a fragment that showed similarity with ferrochelatases but was not identical to the previously isolated CsFeC1 (14, 19). Using this fragment as a probe, a phage clone containing the putative full-length cDNA was obtained (accession number AB037113). The clone was 2,032 bp in length and included a putative open reading frame encoding a polyprotein of 522 amino acids.

The deduced amino acid sequence of this cDNA was compared with those of CsFeC1 and other ferrochelatases. The sequence showed significant similarity with the sequences of other ferrochelatases (Fig. 1A), confirming that it encoded ferrochelatase. This isoform is henceforth referred to as CsFeC2. CsFeC2, like CsFeC1, has an N-terminal extension, which is likely to be a chloroplast transit peptide (see below). CsFeC2 is more similar to AtFC-II (76.2%) than CsFeC1 (58.7%). Phylogenetic analysis of ferrochelatases in photosynthetic organisms showed that CsFeC2, AtFC-II, and ferrochelatases from potato and rice comprised a phylogenetic group different from that of CsFeC1 (Fig. 1B). Moreover, although a cyanobacterial ferrochelatase showed the same level of similarity to CsFeC1 and CsFeC2, the C-terminal region was highly similar to CsFeC2, confirming that it encoded ferrochelatase.

The C-terminal region of CsFeC2 showed similarity to the consensus sequence of light-harvesting chlorophyll-binding proteins (LHCPs), the so-called “generic LHC motifs” (25). All other ferrochelatases in this group, except that from potato, have this motif. Inspection of the potato sequence reveals that it has half of the LHC motif, and if the coding frame is shifted by changing the A triplet of 1416–1418 in the nucleotide sequence into an A doublet, the complete motif is restored. Therefore, it is likely that the potato ferrochelatase sequence contains an error, and all members of this group of ferrochelatases
contain the complete LHC motif. In contrast, the motif is absent from AtFC-I, CsFeC1, and barley ferrochelatase (14). As shown in Fig. 1C, the LHC motif is also conserved in small Cab-like proteins of cyanobacteria (ssl and ssr series), early light-inducible proteins of soybean and Arabidopsis, and high-light inducible proteins (HLIPs) of Synechococcus PCC 7942 (7942 HLIP). All LHCPs contain three membrane-spanning helices, and helices I and III share this generic LHC motif. These helices form the core of the complex and are kept together by ion bridges between the helices (25). Early-light inducible proteins also contain two LHC motifs, whereas HLIPs have only one. These proteins are thought to form homodimers in the thylakoid membrane with two LHC motifs as a core (25, 26). Indeed, in CsFeC2, it is likely that this region was also associated with membrane binding and complex formation (see below).

**Import in Vitro of CsFeC Precursors into Isolated Pea Organelles**—Both CsFeC1 (14) and CsFeC2 have N-terminal extensions of about 90 and 100 residues, respectively, and these are likely to be targeting peptides. To verify this, we performed *in vitro* transport experiments. The 35S-labeled precursors of CsFeC1 and CsFeC2 of 57 and 58 kDa, respectively, (Fig. 2A, TP) were incubated with isolated pea chloroplasts, followed by treatment with thermolysin (pC). It is clear that both precursors were imported into chloroplasts because of the cleavage of the precursors into the same size as that predicted for the mature protein and the disappearance of bands in the presence of Triton X-100 (data not shown). Fractionation of the chloroplasts demonstrated that both mature ferrochelatases were found mainly in the thylakoids. CsFeC1 is clearly also present in the envelope fraction at about 10% of the level in the thylakoids (data not shown). CsFeC2 is also detectable in the envelopes, but at a level much less than that of CsFeC1. The targeting to and distribution within chloroplasts of the two CsFeCs were thus essentially identical to those of the two ferrochelatases from *Arabidopsis* (17).

The experiments from the import of the two radiolabeled CsFeC precursor proteins with isolated pea mitochondria. Again, the two cucumber ferrochelatases behaved in a manner similar to their homologues from *Arabidopsis*. CsFeC2 is not imported (data not shown), whereas CsFeC1 is imported into mitochondria and processed to a protein essentially identical to that imported by chloroplasts (Fig. 2B). This import is abolished by the addition of valinomycin (data not shown), which collapses the membrane potential (27). It has recently been demonstrated that the mitochondrial import system in *vitro* is not robust because it will readily import a number of precursors that are found exclusively in the chloroplast in *vivo*. Therefore, it is not possible to extrapolate the location of a protein in *vivo* from the results of a mitochondrial import experiment. Nevertheless, it was a useful test to discriminate between the two classes of ferrochelatase. This result lends further support to the classification of higher plant ferrochelatases into two separate classes, as does the observation that the barley ferrochelatase (14), which does not have the LHC motif, is imported into mitochondria in a similar fashion as AtFC-I and CsFeC1.

**Expression of CsFeC2 in E. coli and Preparation of Anti-CsFeC2 Antibody**—The putative mature region of CsFeC2 was fused with GST and overexpressed in *E. coli*. The recombinant protein obtained was used for antibody preparation. Unlike CsFeC1 (19), the recombinant GST-CsFeC2 did not show any ferrochelatase activity even after modification of induction and isolation procedures. In addition, the recombinant GST-CsFeC2 protein was bound to glutathione-Sepharose resin for affinity purification, it was not eluted from the resin by 5 mM reduced glutathione or thrombin protease treatment. The fusion protein was therefore recovered from the resin under denaturing conditions, and the GST region was then removed by thrombin treatment. Determination of the N-terminal amino acids of the purified protein confirmed that the obtained recombinant protein was CsFeC2 (data not shown). The molecular mass of the expressed protein calculated from the amino acid sequence was 47.2 kDa and that estimated by SDS-PAGE was 43 kDa. A rabbit was immunized with the purified CsFeC2, and the antibodies were purified by the epitope selection method. The obtained antibodies could detect 1 ng of CsFeC2 or 50 ng of CsFeC1 (data not shown) and were used for Western blotting analyses.

**Immunolocalization of CsFeC2**—To further investigate the subcellular location of CsFeC2, mitochondria and subfractions of chloroplasts were isolated from cucumber cotyledons. The purity of the subcellular fractions from cucumber was checked by Western blotting analysis or enzyme assay for marker proteins (anti-LHCP antibody for thylakoid, anti-large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase antibody for stroma, and fumarase activity for mitochondria). Western blotting analysis of subcellular fractions showed that the anti-CsFeC2 antibody reacted only with the protein in chloroplasts (Fig. 3A). The detected band was similar in size to recombinant CsFeC2, indicating that the artificial cleavage site of recombinant CsFeC2 was very close to the native cleavage site. When chloroplasts were separated into subfractions, the antibody reacted with a thylakoid membrane protein, but not with stromal proteins (Fig. 3A). A band was also detected in the envelope fraction. Because the envelope membrane fraction was somewhat contaminated with thylakoid membranes by our purification procedure, we estimated the extent of the contamination of thylakoid membrane in the envelope membrane using anti-LHCP antibodies (Fig. 3B). From the band intensity of the LHCP protein in the envelope, 10 μg of the envelope fraction was contaminated with ~0.6 μg of thylakoid membranes. Because the band of CsFeC2 was detected in 10 μg of the envelope fraction but not below 5 μg of thylakoid membranes, the detected band in the envelope membrane was not due to the contaminating thylakoid membranes. Consistent with the results of the *in vitro* transport experiment, no band of CsFeC2 was detected in the mitochondrial fraction. Thus, it is con-
cluded that CsFeC2 functions for heme biosynthesis in chloroplasts, but not in mitochondria.

**Formation of Complexes of CsFeC2 in the Thylakoid Membrane**—We then examined the association of CsFeC2 with thylakoid membranes. CsFeC2 protein in the thylakoid membrane was not eluted by salt or alkaline treatment, indicating that CsFeC2 was not associated with thylakoid membranes by electrostatic forces (data not shown). The hydrophathy profile of CsFeC2 indicated that the obvious hydrophobic region was only recognized in the C-terminal region containing the LHC motif (data not shown). Therefore, like LHCPs, members of the CsFeC2 protein family are probably bound to the thylakoid membrane through this C-terminal LHC motif. To investigate the formation of a CsFeC2 complex, we treated isolated thylakoid membranes with different concentrations of SDS (0.4–2%), and chlorophyll-protein complexes were separated in a slab gel containing 0.1% SDS. The untreated thylakoid membranes could not be separated in the gel. However, after treatment with 0.4–2% SDS, chlorophyll-binding proteins migrated with chlorophyll (data not shown). Western blotting analysis with anti-CsFeC2 antibody indicated that CsFeC2 did not comigrate with major chlorophyll-binding proteins, but formation of two complexes, complex I and complex II, was observed at low SDS concentrations (Fig. 4A). To confirm that these complexes originated from CsFeC2, they were eluted from the gel slices, treated by boiling in SDS sample buffer, and subjected to SDS-PAGE. Western blotting analysis revealed that both complexes contained CsFeC2 (Fig. 4B). These complexes were dissociated into monomers by increasing the SDS concentration (Fig. 4A). Boiling the sample in 2% SDS completely dissociated the complexes to monomers. The standard molecular mass was estimated using a LMW marker kit (Amersham Biosciences, Inc.) treated at 4°C in buffer containing 0.4% SDS. The molecular masses of the complexes were estimated to be about 110–120 kDa (complex I) and about 90–95 kDa (complex II), although the mobility of partially denatured proteins shows some differences from that of completely denatured proteins. Because the molecular mass of the CsFeC2 monomer was estimated to be about 50 kDa in this partially denaturating system (the molecular mass of CsFeC2 estimated in Fig. 4 was about 43 kDa), it is possible that these complexes were CsFeC2 homodimers with slightly different conformations that caused the slight differences in mobility. Alternatively, these complexes might contain component(s) other than CsFeC2, for instance, some pigment molecules (see “Discussion”).

**Expression of Two Types of Ferrochelatases in Cucumber**—We investigated the differences in expression between the two ferrochelatase genes (CsFeC1 and CsFeC2). As reported previously (19), CsFeC1 mRNA was accumulated in nonphotosynthetic tissues such as the roots and hypocotyls (Fig. 5). On the other hand, CsFeC2 mRNA was detected in all tissues of seedlings. Moreover, CsFeC2 mRNA was markedly induced by illumination in cotyledons, in contrast to the light-insensitive expression of CsFeC1. We also investigated the expression in mature leaves and flowers (Fig. 5). The CsFeC1 gene was expressed in flowers but not in leaves, whereas the CsFeC2 gene was expressed in both of these tissues. These light- and tissue-dependent expression profiles of CsFeC genes were reminiscent of the similar regulation of two distinctly controlled hemA genes (hemA1 and hemA2) encoding glutamyl-tRNA reductase in cucumber (28). Glutamyl-tRNA reductase catalyzes the initial step of 5-aminolevulinic acid
biosynthesis and is considered to be the rate-limiting step of total tetrapyrrole biosynthesis. It was reported that hemA1 mRNA was light-inducible and primarily detected in cotyledons and hypocotyls, whereas hemA2 mRNA was poorly light-responsive and was detected in roots and hypocotyls but hardly detected in cotyledons (28). The similarity of the expression patterns between the hemA and ferrochelatase genes suggested that there are two pathways for tetrapyrrole biosynthesis in higher plants, i.e., one pathway for heme biosynthesis in non-photosynthetic tissues and the other for Chl and heme biosynthesis in the photosynthetic organelles, the chloroplasts. Interestingly, when cucumber cotyledons were treated with cycloheximide, a cytoplasmic translational inhibitor, CsFeC1 and hemA2 mRNAs were markedly induced (Fig. 6), whereas those of CsFeC2 and hemA1, which are transcribed in photosynthetic tissues, were not affected. This suggested that the expression and/or accumulation of CsFeC1 and hemA2 gene products are controlled by the same regulatory mechanism.

DISCUSSION

We isolated a CsFeC2 cDNA from the cDNA library of cucumber cotyledons and demonstrated immunologically that CsFeC2 is a membrane-spanning protein located mainly in the thylakoid membrane. The CsFeC2 protein is presumed to have evolved from cyanobacterial ferrochelatase, which possesses the C-terminal generic LHC motif. Jansson (25) suggested that the ancient ferrochelatase captured a membrane-spanning helix from a HLIP to fulfill functions for membrane anchoring or photoprotection of porphyrins. In fact, CsFeC2 is likely to be anchored to the thylakoid membrane with the generic LHC motif. Moreover, it is also likely that CsFeC2 protein forms a homodimer (complex II) in the thylakoid membrane, probably via the generic LHC motif. In fact, HLIPs and stress-enhanced proteins containing only one LHC motif have been predicted to form homodimers (25, 29). Because HLIPs are thought to bind chlorophylls, it is possible that CsFeC2 also binds pigments with its LHC motif. Funk and Vermaas (30) discussed the possibility that the LHC domain of Synechocystis ferrochelatase binds chlorophyll. We found that complex I of CsFeC2 has a slightly (10–20 kDa) higher molecular mass than complex II that probably corresponds to a homodimer of CsFeC2. It is attractive to hypothesize that some pigment molecules bind to complex I, resulting in the higher molecular mass of complex I.

Matringe et al. (11) reported that in pea chloroplast, ferrochelatase activity was associated with the thylakoid membrane only, and no activity was detected in the envelope membrane. However, the anti-CsFeC2 antibody cross-reacted with protein in the envelope membrane. The envelope localization of both Arabidopsis ferrochelatases was also reported after import in vitro into pea chloroplasts (17). These results suggest that there is no clear spatial separation of the chelatases within chloroplasts (17), in contrast to the previous proposal to explain the control of flux between the chlorophyll and heme branches of the pathway (11). However, the flux of protoporphyrin IX to chlorophyll synthesis in the envelope may be much larger than that to heme synthesis because the amount of ferrochelatase in the envelope was very small, and the affinity of Mg-chelatase for protoporphyrin IX was reported to be much higher than that of ferrochelatase (31). One possible function of this envelope ferrochelatase is the biosynthesis of hem to be distributed to the chloroplast or cytoplasm. Although the organelle from which heme is supplied to cytosolic heme proteins has not been clarified, heme efflux was observed from isolated chloroplasts (32). Another possibility is that the envelope-localized ferrochelatase may act as a scavenger, mopping up any excess protoporphyrin IX in the envelope before this highly reactive compound causes photo-oxidative damage.

The CsFeC2 gene expression profile was quite different from that of the CsFeC1 gene. Compared with CsFeC1, CsFeC2 mRNA was markedly induced by light in cotyledons, suggesting that the expression was induced simultaneously with chloroplast development. Because the induction is consistent with the appearance of cytochrome f (33) and probably of cytochrome bc1, the heme prosthetic group of these proteins may be supplied by CsFeC2.

The similar expression profiles of respective isoforms of ferrochelatases and glutamyl-tRNA reductases (hemA) in cucumber suggest that there are two separate pathways of tetrapyrrole biosynthesis with two isozymes in each step. The pathway in photosynthetic tissues seems to be localized to the chloroplast, and the steps toward protoporphyrin IX may be shared by Chl and heme synthesis. On the other hand, the pathway in nonphotosynthetic tissues was not affected by light and may supply heme for hemoproteins or for biosynthesis of the phytochrome chromophore. The expression of the genes for CsFeC1 and hemA2, localized in nonphotosynthetic tissues, were both induced in photosynthetic tissues by cycloheximide treatment. The cycloheximide induction may be caused by a putative factor(s) required for repression of CsFeC1 and hemA2 expression specifically in photosynthetic tissues.

In summary, we have demonstrated the existence of two ferrochelatases in cucumber that are controlled distinctly, and we have shown that the ferrochelatase expressed in photosyn-
thetic tissue was located in chloroplasts. Regulation of distinct pathways of tetrapyrrole biosynthesis in photosynthetic and nonphotosynthetic tissues should be further analyzed.

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