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The Arabidopsis Photomorphogenic Mutant *hy1* Is Deficient in Phytochrome Chromophore Biosynthesis as a Result of a Mutation in a Plastid Heme Oxygenase

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The *HY1* locus of Arabidopsis is necessary for phytochrome chromophore biosynthesis and is defined by mutants that show a long hypocotyl phenotype when grown in the light. We describe here the molecular cloning of the *HY1* gene by using chromosome walking and mutant complementation. The product of the *HY1* gene shows significant similarity to animal heme oxygenases and contains a possible transit peptide for transport to plastids. Heme oxygenase activity was detected in the HY1 protein expressed in *Escherichia coli*. Heme oxygenase catalyzes the oxygenation of heme to biliverdin, an activity that is necessary for phytochrome chromophore biosynthesis. The predicted transit peptide is sufficient to transport the green fluorescent protein into chloroplasts. The accumulation of the HY1 protein in plastids was detected by using immunoblot analysis with an anti-HY1 antiserum. These results indicate that the Arabidopsis *HY1* gene encodes a plastid heme oxygenase necessary for phytochrome chromophore biosynthesis.

INTRODUCTION

Light is one of the most important environmental factors and regulates many processes involved in plant growth and development. Plants have at least three families of photoreceptors that specifically recognize different wavelengths of light: the red(R)/far-red(FR)–sensing phytochrome family, blue(B)/UV-A photoreceptors termed cryptochromes, and UV-B photoreceptors (Kendrick and Kronenberg, 1994). Of these three, the phytochrome family has been characterized the most extensively (Quail, 1991; Smith, 1995). Holophytochrome is composed of an apoprotein of ~1100 amino acids to which a linear tetrapyrrole chromophore is covalently attached. It exists in either of two photointerconvertible forms: the R light–absorbing (Pr) form or the FR light–absorbing (Pfr) form.

Two pathways are involved in phytochrome biosynthesis. One is for phytochrome apoproteins, which are synthesized by a nuclear-encoded multigene family (Sharrock and Quail, 1989; Clack et al., 1994), and the other is for the chromophore (Lagarias and Rapoport, 1980). The structure of the tetrapyrrole chromophore is thought to be common to all phytochromes. The pathway for phytochrome chromophore biosynthesis shown in Figure 1 is based on metabolic studies (Terry et al., 1993). 3(*E*)-Phytochromobilin (PΦB) is attached to apophytochrome (Cornejo et al., 1992; Terry et al., 1995) by an autocatalytic process (Elich and Lagarias, 1989; Lagarias and Lagarias, 1989). The proposed biosynthesis pathway for PΦB is common to chlorophyll synthesis from 5-aminolevulinic acid to protoporphyrin IX. The pathway branches from chlorophyll biosynthesis at the point of conversion of protoporphyrin IX to heme by ferrochelatase. Heme is oxidized to biliverdin (BV) IXα, which is reduced to 3(*Z*)-PΦB and subsequently isomerized to PΦB (Terry et al., 1995). Although the pathway is relatively well understood, none of the genes (except for the one encoding ferrochelatase) encoding biosynthesis enzymes in the pathway after branching from chlorophyll (Smith et al., 1994) has been cloned.

Molecular genetic approaches have contributed to the understanding of photomorphogenesis in plants (Chory et al., 1996). One class of photomorphogenic mutants shows a partially etiolated morphology when grown in light. In Arabidopsis, *hy* long hypocotyl mutants have been isolated and characterized (Koornneef et al., 1980). Mutants for phytochrome A, such as *hy8* (*phyA*), and for phytochrome B, such

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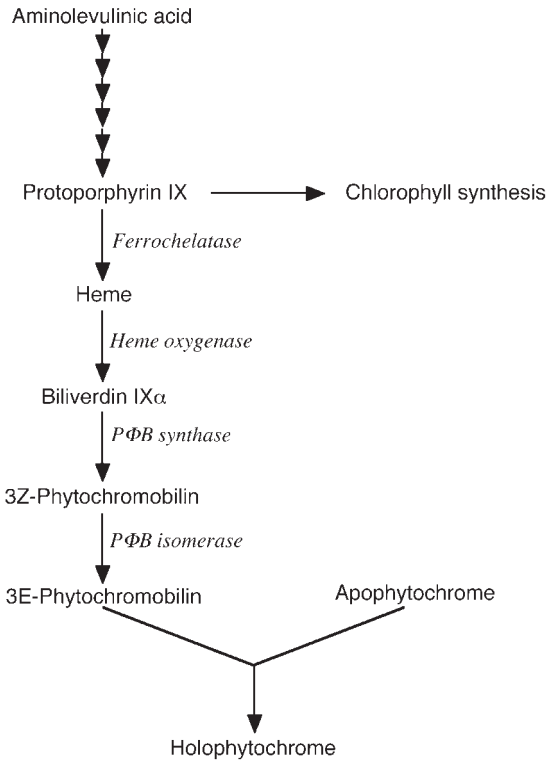

Figure 1. Proposed Pathway for Phytochrome Chromophore Biosynthesis in Plants.

This model was adapted from that given in Terry et al. (1993).

as *hy3* (*phyB*), have contributed to studies of the physiological roles of phytochrome (Nagatani et al., 1993; Reed et al., 1994; Shinomura et al., 1994, 1996). The *hy1* and *hy2* mutants do not respond to R and FR light, although the phytochrome apoprotein is synthesized normally (Chory et al., 1989). These mutants are probably deficient in phytochrome chromophore biosynthesis, because feeding experiments with biliverdin restore the wild-type phenotype (Parks and Quail, 1991). Similar mutants are *aurea* (*au*) and *yellow-green-2* in tomato (Terry and Kendrick, 1996; van Tuinen et al., 1996) and *pcd1* and *pcd2* (for *phytochrome chromophore deficient*) in pea (Weller et al., 1996, 1997). The tomato mutant *au* is often used for studies on gene expression, physiology, and signaling mediated by phytochrome (Oelmüller and Kendrick, 1991; Becker et al., 1992; Bowler and Chua, 1994). These mutants are also useful in studies of the biochemistry and physiology of phytochrome chromophore biosynthesis (Parks and Quail, 1991; Terry, 1997).

Here, we report the map-based cloning of the Arabidopsis *HY1* gene and show that it encodes a plastid-located heme oxygenase. The molecular cloning of *HY1* will contribute not only to further characterization of phytochrome chromophore biosynthesis but also to an understanding of photomorphogenesis in plants, because these mutants are often used as phytochrome-deficient plants in physiological and biochemical studies.

RESULTS

Chromosome Walking to *HY1*

To determine the primary function of the HY1 protein, we have isolated the *HY1* gene by using a map-based cloning strategy. Fine-structure mapping of *HY1* was performed using two double mutants that, in addition to *hy1*, have another visible mutation on chromosome 2, namely, *hy1 er* and *hy1 as.* These double mutant lines in the Landsberg background were crossed to wild-type Columbia plants. The F_1 progeny were, as expected, phenotypically wild type, because all of the mutations are recessive. Those F_2 progeny having a recombinational breakpoint in the vicinity of *HY1* were collected, and the breakpoints were mapped using F_3 DNA from these plants (see Table 1) and restriction fragment length polymorphism (RFLP) markers on chromosome 2 (Chang et al., 1988; Nam et al., 1989).

A yeast artificial chromosome (YAC) contig for the *HY1* region of chromosome 2 (Figure 2A) was constructed from the EG (Grill and Somerville, 1991) and EW (Ward and Jen, 1990) libraries by using the RFLP marker *GPA1* (an Arabidopsis G protein α subunit gene) as the starting point (Ma et al., 1990). The end fragments of each YAC DNA insert were rescued by inverse polymerase chain reaction (PCR) (Ochman et al., 1988) and used as probes for establishing the identity of neighboring YACs and for further RFLP mapping. Based on their map positions, two YACs, EW20C4 and EG12B5-2, were identified as candidates for containing the *HY1* gene. The yeast clone EG12B5 contains two YACs in a single cell; we named the smaller YAC EG12B5-2. Subgenomic libraries in λ FixII were made from YACs EG12B5-2 and EW20C4. Physical mapping of the λ clones was performed to develop a fine structure physical map in the *HY1* region, and the λ clones were also used to search for new RFLPs in the region. During the course of this work, a new codominant cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) marker, ks450, was identified and also used for mapping (Table 1 and Figure 2A). The minimum region identified genetically as containing *HY1* was ~60 kb.

Complementation of the *hy1* Mutation in Transgenic Plants

A genetic complementation test was conducted by introducing wild-type cloned genomic DNA into mutant plants. Insert fragments of λ clones were subcloned into the binary

vector pBIN19, and the T-DNA was introduced into the *hy1* mutant by Agrobacterium-mediated transformation (Valvekens et al., 1988). The regenerants (T_1 generation) were self-fertilized, and their progeny were sown on plates containing kanamycin to confirm that they contained T-DNA. The hypocotyl lengths and cotyledon colors of 30 to 50 plants from each of these T_2 families were measured. A 20-kb genomic fragment in λ clone 79 rescued the *hy1*-conferred phenotype when introduced into the *hy1* mutant (Figures 3A and 3B).

Further experiments were performed with T_3 generation transgenic plants to test whether the phenotypic rescue was associated with restoration of a functional phytochrome. Photoreversibly functional phytochrome cannot be detected in *hy1* mutants; therefore, we assayed for photoreversibility of phytochrome by using difference spectrum for in vitro phytochrome phototransformation in etiolated transformants (Figure 4). Rescued plants have photoreversibile phytochrome, whereas no photoreversibility of the phytochrome was detected in the *hy1* mutant. These analyses confirmed that λ clone 79 can correct the defect of the *hy1* mutation.

Identification and Sequence Analysis of the *HY1* Gene

The results of the complementation test indicated that *HY1* is located in the genomic region represented in λ clone 79. To help to identify the *HY1* gene, we isolated expressed genes in the corresponding genomic region from a cDNA library by using genomic fragments as probes. Seventeen cDNAs were isolated, and their nucleotide sequences were partially determined from their 5′ ends. The isolated cDNAs fell into three groups (a, b, and d; Figure 2B). Although it is possible that these cDNAs do not account for all of the expressed genes in this genomic region, we determined the complete nucleotide sequence for the largest cDNA clone in each of the three cDNA groups and analyzed the encoded proteins by comparing their amino acid sequences with those of other proteins in the databases. All showed similarity or identity to genes in the databases. The gene product for the cDNA in group a (Figure 2B) is related to a cDNA for β-ketoacyl–coenzyme A synthase in canola (Lassner et al., 1996). The gene product for the cDNA in group b (Figure 2B) is identical to the Arabidopsis *AKT1* gene, which encodes a potassium transporter (Sentenac et al., 1992). The predicted product of the cDNA in group d (Figure 2B) shares significant sequence identity with animal heme oxygenase. Heme oxygenase catalyzes the synthesis of BV IXα from heme (Figure 1), and this step is predicted to be involved in the biosynthesis of the phytochrome chromophore in plants (Terry et al., 1993). Therefore, this gene (d) seemed to be a good candidate for *HY1.*

To confirm the identification of *HY1*, we determined and compared the nucleotide sequences for the cDNAs and genomic DNAs from various wild-type and *hy1* mutant alleles. Genomic clones corresponding to the wild-type *HY1* cDNA were isolated from a genomic library of Columbia by using a group d cDNA as a probe. The nucleotide sequence of the genomic clone was determined (∼4 kb) (GenBank accession number AB021857). The gene showing similarity to heme oxygenase consists of three exons and two introns

Table 1. Recombination between *HY1* and Selected Molecular Markers on Chromosome 2

Molecular Marker	Enzyme Used for RFLP Analysis	Number of Recombinants	Total Number of Plants Examined	Relative Distances[a]
m283[b]	BglII	19	58	0.328[c]
m220[b]	SacII	8	34	0.235[c]
EG12B5-2R[d]	EcoRI	1	92	0.011[c]
ks450[e]	HpaII	2	206	0.010[c]
EW7G5L[d]	HindIII	0	92	0.000[c]
				from *ER* to *HY1*[a]
GPA1[f]	SpeI	8	8	1[g]
EG10A10R[d]	BglII	5	8	0.625[g]
EG16C6R[d]	NsiI	5	8	0.625[g]
EW20C4L[d]	ClaI	5	8	0.625[g]
EW7G5L[d]	HindIII	1	8	0.125[g]
EF12B5-2R[d]	EcoRI	0	8	0.000

[a]Number of recombinants per total number of plants examined.
[b]RFLP marker (Chang et al., 1988).
[c]From *AS* to *HY1*.
[d]End fragment of YAC insert. R is the right end fragment, and L is the left end fragment.
[e]A new CAPS marker found during this study.
[f]Ma et al. (1990).
[g]From *ER* To *HY1*.

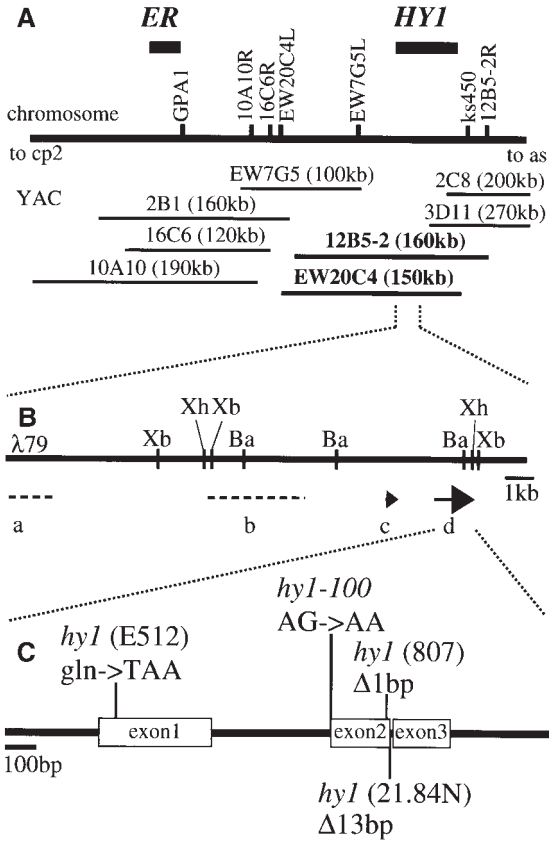

Figure 2. Positional Cloning of the *HY1* Gene.

(A) Genetic and physical map in the vicinity of the *HY1* locus on chromosome 2. A YAC contig for the HY1 region of chromosome 2 was constructed from the EG (Grill and Somerville, 1991) and EW libraries (Ward and Jen, 1990) by using the RFLP marker *GPA1* as the starting point (Ma et al., 1990). The YAC names without a prefix are EG clones, and R and L designate the right and left end fragments isolated by inverse PCR, respectively; for example, 10A10R is the right end fragment from EG10A10. EW clones are as marked. The positions of the RFLP and CAPS markers are indicated by vertical lines. Horizontal lines depict the YAC clones, and their approximate lengths in kilobases are within parentheses.

(B) Map of the *HY1* region in λ clone 79 (λ 79). Three genes (a, b, and d) isolated by cDNA library screening and one gene (c) deduced from nucleotide sequencing are shown. The proteins encoded by these genes are as follows: a, β-ketoacyl–coenzyme A synthase homolog; b, potassium channel protein AKT1; c, Ids-4 homolog; and d, heme oxygenase homolog. Ba, BamHI; Xb, XbaI; Xh, XhoI.

(C) Gene organization deduced from nucleotide sequencing and mutations in the *HY1* gene.

(Figure 2C). In the upstream region of the genomic clone, another gene (c) that had not been isolated in our cDNA screening was identified (Figure 2B). It is oriented in the same direction as the *HY1* gene and shows similarity to a gene encoding the Ids-4 (for iron deficiency–specific) protein, which is induced by iron deficiency in barley (EMBL accession number D14161).

The genomic DNA fragments for the gene showing similarity to heme oxygenase were amplified by using PCR. The fragments were from Landsberg *erecta* and the *hy1* mutants. Their nucleotide sequences as well as sequences from reverse transcription–PCR products from the wild type and mutants were determined. A comparison of the mutant and wild-type sequences indicates that all of the *hy1* mu-

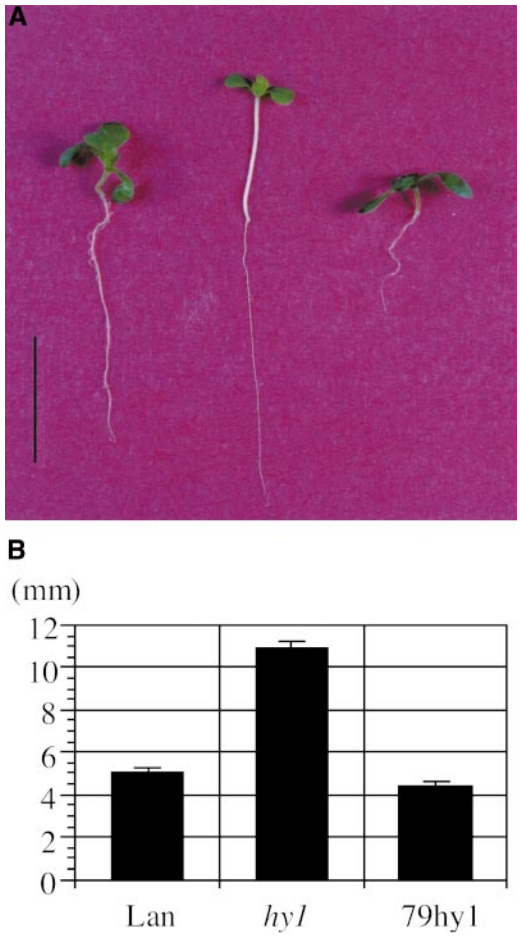

Figure 3. Complementation of the *hy1* Mutation in Transgenic Plants.

(A) Nine-day-old seedlings grown under long-day conditions (16 hr of light and 8 hr of dark) were photographed. From left to right, the plants are Landsberg *erecta* wild type, the *hy1*(21.84N) mutant, and a transgenic *hy1* plant transformed with a pBIN19 construct containing insert DNA in λ clone 79. Bar = 10 mm.

(B) Hypocotyl length of each plant line. Error bars represent standard error. Lan, Landsberg *erecta*; *hy1*, the *hy1*(21.84N) mutant; 79hy1, the complemented transformed plant.

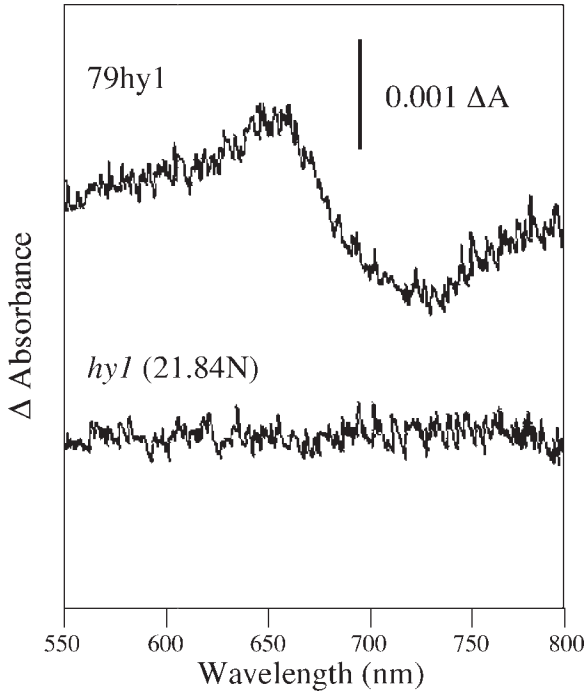

Figure 4. Difference Spectra of Crude Extracts from Etiolated Seedlings.

Difference spectra after FR actinic irradiation were recorded relative to absorption spectra before actinic irradiation of crude extracts from the *hy1*(21.84N) mutant and from the complemented transformed plant (79hy1).

tants tested have a sequence change (Figure 2C). The *hy1*(21.84N) mutant has a 13-base deletion (encompassing the end of the second exon and the first nucleotide in the second intron) that causes a frameshift. The *hy1 as ER*(807) allele has a one-nucleotide deletion in the second exon, causing a frameshift and possibly synthesis of truncated peptides. The *hy1*(E512) allele has a nucleotide substitution from C to T, converting a CAA codon (Gln) to a TAA stop codon. We also analyzed the *hy1-100* mutant (previously distributed as *hy6* seeds from the Arabidopsis Biological Resource Center, Columbus, OH). The *hy1-100* mutant has an AG-to-AA substitution in the normally conserved sequence at the acceptor site (3′ boundary) of the first intron that presumably leads to a splicing defect.

To confirm the gene identity of *HY1*, we conducted RNA gel blot analysis of the wild type and the *hy1* mutants. Accumulation profiles of the *HY1* transcript from the wild type and the *hy1* mutants were compared (Figure 5A). The level of the *HY1* transcripts in all mutants was slightly reduced. A signal (of the size expected for an unspliced RNA) was also observed in the *hy1-100* mutant (Figure 5A). This RNA in *hy1-100*, at the size expected for the mature mRNA, appears to correspond to a misspliced RNA molecule detected by reverse transcription–PCR analysis (data not shown). Therefore, *hy1-100* is probably a "splicing mutant," with inefficient utilization of the AA mutant acceptor site at the correct position or use of an incorrect proximal AG. We also analyzed the amount of the HY1 protein that accumulated in the four *hy1* mutants (Figure 5B), using an antibody raised against recombinant HY1 synthesized in *Escherichia coli.* Although protein profiles on SDS-PAGE stained by using Coomassie Brilliant Blue R 250 were basically similar to each other in the wild type and mutants, no HY1 protein was detected by the anti-HY1 antiserum in any *hy1* mutant (Figure 5B).

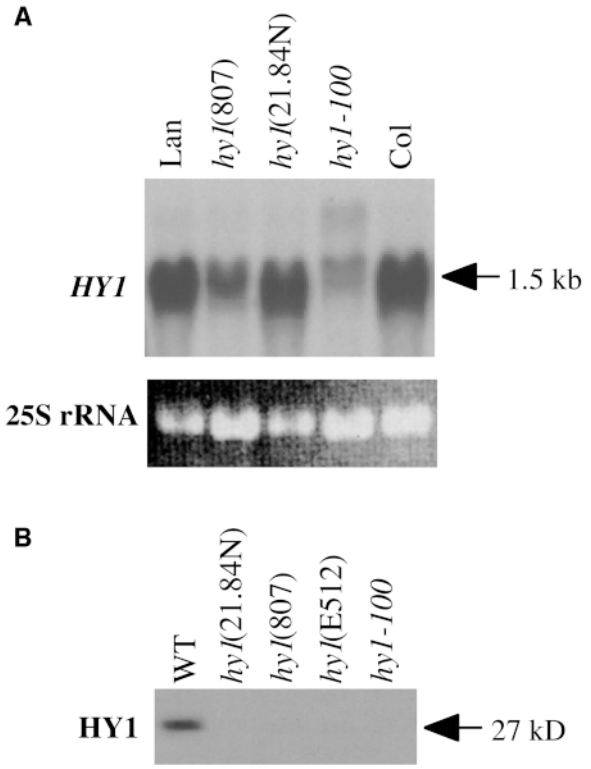

Figure 5. Detection of Transcripts and Protein in *hy1* Mutants.

(A) RNA gel blot analysis. Total RNA (10 μg) from wild-type Columbia (Col) and Landsberg *erecta* (Lan) plants and various *hy1* mutants was analyzed by RNA gel blotting using the *HY1* cDNA as a probe. The length of the transcript is indicated at right in kilobases. Ethidium bromide staining of the 25S rRNA was used as a loading control.

(B) Immunoblot analysis. Extracts from wild-type Columbia (WT) and from various *hy1* mutants were analyzed by protein gel blotting using an antibody raised against recombinant HY1—the product of gene d (see Figure 2C) expressed in *E. coli.*. The molecular mass of the protein is indicated at right in kilodaltons.

These data, together with the mapping and complementation results, indicate that the gene encoding the protein with similarity to heme oxygenase is *HY1.*

Similarity of the *HY1* Gene Product to Heme Oxygenase

The HY1 protein is similar to heme oxygenase in size and structure. The *HY1* gene encodes a single open reading frame of 282 amino acids with a predicted molecular mass of 32.6 kD. The deduced amino acid sequence shows weak similarity to animal heme oxygenases (Figure 6). Indeed, the heme oxygenase signature sequence LLVAHAYTR (amino acid residues 128 to 136 in animal heme oxygenases), which is highly conserved among heme oxygenases of several animal species (Sun and Loehr, 1994; Takahashi et al., 1994a, 1994b, 1995; Ito-Maki et al., 1995), is also conserved in HY1 (Figure 6). In particular, H-132 in animal heme oxygenase-1, which is thought to play a structural role in stabilizing the heme oxygenase protein (Matera et al., 1997), is maintained in HY1. In addition, H-86 in HY1, which corresponds to H-25 of heme oxygenase-1 in animals (Ito-Maki et al., 1995), is also conserved. H-25 is the axial heme iron ligand and is therefore an important residue for enzyme activity (Ito-Maki et al., 1995). The conservation of these important residues suggests that the sequence similarity of HY1 to animal heme oxygenase is significant.

In animal heme oxygenases, the C-terminal hydrophobic amino acid sequence is important for microsomal localization. The corresponding domain is absent in the predicted Arabidopsis HY1 protein. However, tryptic digestion of the membrane binding C-terminal domain produces a catalytically active 28-kD water-soluble form (Yoshida et al., 1991), that is, this hydrophobic domain is only needed for microsome localization in animals. By analogy, the absence of the corresponding region in HY1 should not be essential for its function as a heme oxygenase. The HY1 protein has a 55–amino acid sequence at its N terminus that is a possible transit peptide for localization to plastids (von Heijne et al., 1989). This putative chloroplast transit peptide in HY1 is rich in serine (12 of the 55 amino acid residues are serines). Furthermore, the consensus sequence of the cleavage site, $(Val/Ile)_{-3}-X_{-2}-(Ala/Cys)_{-1}-Ala_{+1}$ (Gavel and von Heijne, 1990), was also observed as Val-Val-Ala-Ala at amino acid

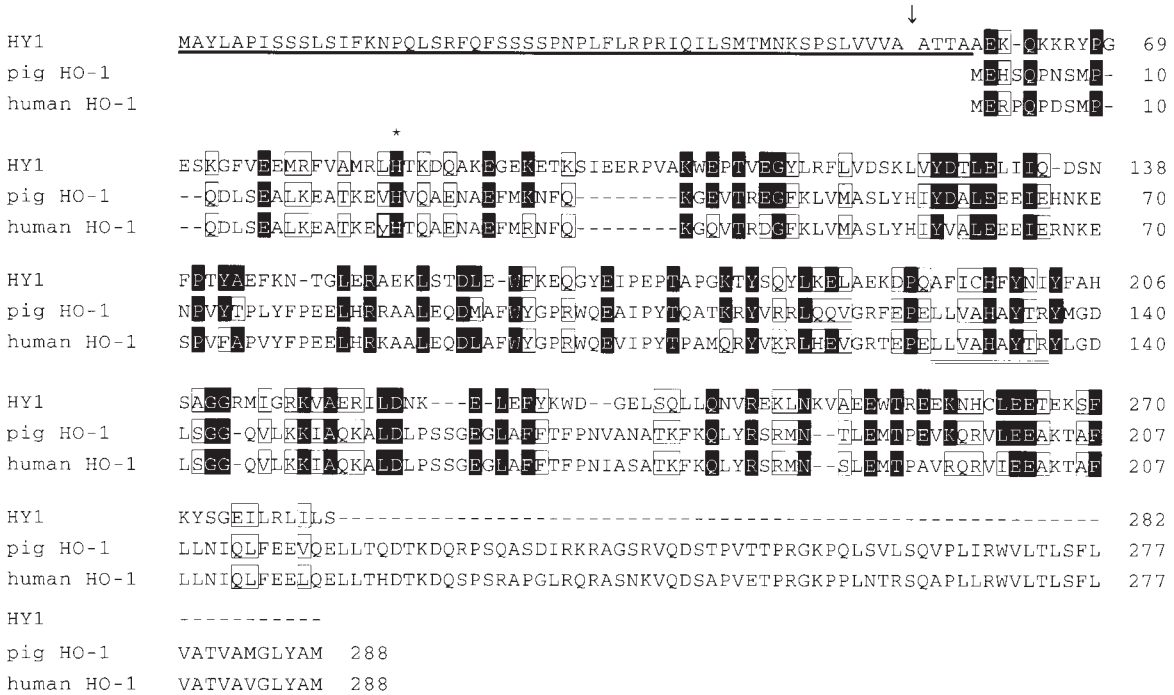

Figure 6. Sequence Comparison of HY1 with Mammalian Heme Oxygenases.

The deduced amino acid sequence of *HY1* was aligned with pig and human heme oxygenase-1 (HO-1; Yoshida et al., 1988; Suzuki et al., 1992). Dashes indicate gaps introduced to maximize alignment. Identical amino acid residues (in black boxes) and similar residues (open boxes) are highlighted. The putative transit peptide used for the transport experiment (see Figure 7) is underlined. An arrow indicates a possible cleavage site between the transit peptide and mature protein. The conserved histidine residue, which is important for the interaction with heme, is shown by an asterisk (Ito-Maki et al., 1995). The heme oxygenase signature sequence is double underlined.

residues 53 to 56 in the deduced HY1 protein sequence (Figure 6). The predicted mature HY1 protein is 227 amino acids with a molecular mass of 26.6 kD. Indeed, the immunoreactive HY1 protein has an apparent molecular mass of 27 kD, as observed on protein gel blots (Figure 5).

Heme Oxygenase Activity in the Recombinant HY1 Protein

The HY1 protein without the putative transit peptide (HY1ΔTP) was expressed in *E. coli* by using the pGEX4T-3 vector. The heme oxygenase assay was performed with biliverdin reductase (BVR) as a coupling enzyme. Heme oxygenase activity was detected in crude extracts after the induction of the *HY1* gene by adding isopropyl β-D-thiogalactopyranoside (Figure 7). Figure 7A shows the time-dependent spectral changes in the reaction mixture containing HY1ΔTP fused to glutathione *S*-transferase (GST). The absorbance at 400 nm (λ_{max} of heme) decreased with time as the absorbance at 450 nm (λ_{max} of bilirubin) increased. This indicates that the reaction product is bilirubin IXα. No spectral changes were detected when the crude extract contained only GST (Figure 7B). Therefore, we conclude that the HY1 protein has a heme oxygenase activity.

Subcellular Localization of HY1

The gene for *HY1* was found to encode a possible transit peptide for localization to plastids (Figure 6). The green fluorescent protein (GFP) from jellyfish has been used as a vital reporter in plant cells (Chiu et al., 1996). To test whether the predicted transit peptide was functional, we fused the DNA encoding the putative transit peptide to the *GFP* gene and placed the DNA under the control of the cauliflower mosaic virus 35S promoter. The construct was introduced into tobacco leaves by bombardment. Transient expression was observed by using fluorescence microscopy, and the green fluorescence was localized to chloroplasts in guard cells of the tobacco leaf (Figures 8A and 8B). This suggests that the transit peptide of HY1 is functional and sufficient to transport proteins to plastids and further implies that the *HY1* gene product in plants is most likely localized in the plastids. On the other hand, no subcellular localization of GFP was detected following transient expression from the control GFP without the transit peptide (Figures 8C and 8D).

To further examine the subcellular localization of the HY1 protein, we conducted protein gel blot analysis with the anti-HY1 antiserum using subcellular fractions. Rosette leaves were homogenized and separated into soluble and insoluble fractions by centrifugation. The chloroplast fraction was prepared using a Percoll step gradient. The HY1 protein at 27 kD was detected in the soluble fraction of leaf protein and in the chloroplast fraction (Figure 8E). These data would indicate that HY1 is a soluble plastid protein.

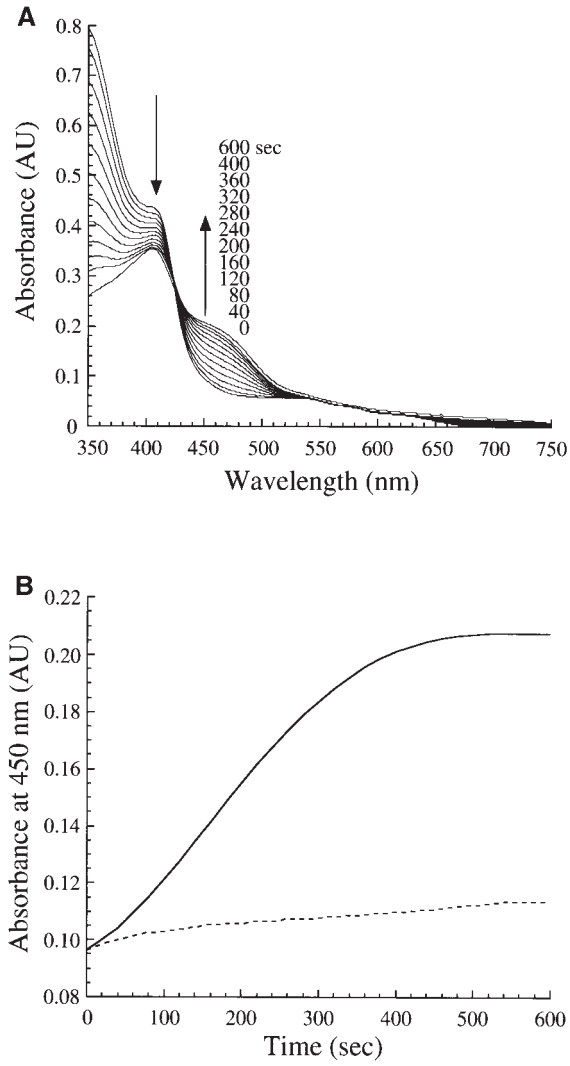

Figure 7. Heme Oxygenase Activity of GST–HY1ΔTP.

(A) Time-dependent spectral change of the reaction mixture. The spectra were recorded immediately after the addition of NADPH (0 sec) and at 40-sec intervals up to 400 and then again at 600 sec. The arrows indicate the direction of absorbance changes during incubation.

(B) Heme oxygenase activity of GST–HY1ΔTP as a function of time. The solid line shows the absorbance changes as a function of time when the *E. coli* cell extract contained GST–HY1ΔTP. The broken line shows the same absorbance changes; however, here the *E. coli* cell extract contained GST. AU, arbitrary units.

DISCUSSION

We have used a map-based strategy to clone the Arabidopsis *HY1* gene that is involved in chromophore biosynthesis of photoreceptor phytochromes. The *hy1* mutation was

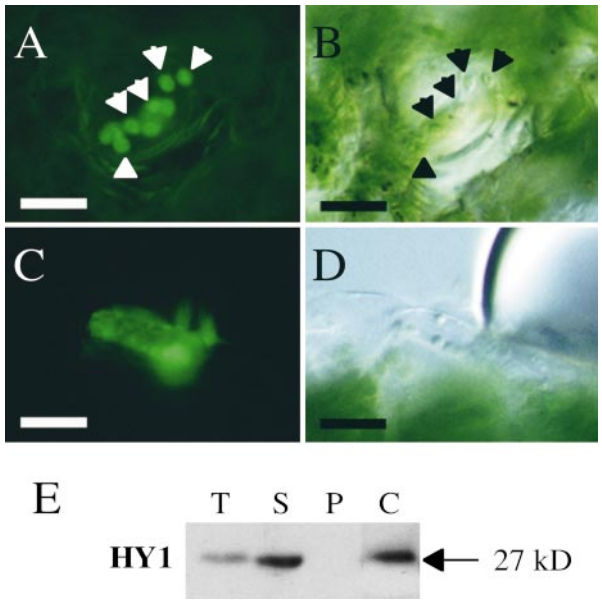

Figure 8. Subcellular Localization of the HY1 Protein.

(A) to **(D)** Distribution of GFP fluorescence in tobacco leaves. Constructs carrying 35SΩ transit peptide (*HY1*)–sGFP(S65T) (**[A]** and **[B]**) and 35SΩ–sGFP(S65T) (**[C]** and **[D]**) were introduced into tobacco leaves. **(A)** and **(C)**, and **(B)** and **(D)** are fluorescent and bright-field images of the cells, respectively. The expression and localization of GFP was observed. In **(B)**, the arrows indicate easily recognizable chloroplasts. In **(A)**, the arrows correspond to the arrows in **(B)**. Bars = 25 μm.

(E) Immunochemical detection of the HY1 protein in fractionated cell extracts. Extracts from leaves and chloroplasts of wild-type Columbia were analyzed by protein gel blotting. T, total protein of wild-type leaves; S, soluble fraction of wild-type leaves; P, insoluble fraction of wild-type leaves; C, chloroplast fraction of wild-type leaves. The molecular mass of the protein is indicated at right in kilodaltons.

complemented by a relatively long genomic DNA fragment contained within λ clone 79. There are at least four genes in this clone. One of the genes shows significant sequence similarity to heme oxygenase. The corresponding gene in all of the *hy1* mutant lines is mutated (Figure 2B). When a cDNA encoding the wild-type heme oxygenase, under the control of the 35S promoter, was introduced into wild-type plants, most of the transgenic lines showed *hy1*-conferred phenotypic traits, such as a long hypocotyl, pale color, and early flowering (T. Muramoto, unpublished observation). This may be due to gene silencing. Therefore, Arabidopsis *HY1* is the gene encoding the HY1 protein that is similar to animal heme oxygenase.

In the heme oxygenation reaction, oxidative removal of the α-methene bridge carbon atom as a carbon monoxide and three successive monooxygenation steps occur to produce BV. Heme and BV are intermediates of the phytochromobilin synthesis pathway (Figure 1). The heme oxygenase reaction is expected to be involved in phytochrome chromophore biosynthesis in plants. Because biochemical and physiological studies have shown that the *HY1* gene product is involved in phytochrome chromophore biosynthesis (Chory et al., 1989; Parks and Quail, 1991), it is reasonable that the gene product of *HY1* shows similarity to a heme oxygenase in animals. The significance of this similarity is supported by the demonstration of heme oxygenase activity in the *E. coli* crude extracts expressing the HY1 protein (Figure 7). Although there is similarity between animal heme oxygenase and the HY1 protein of Arabidopsis, HY1 contains a functional transit peptide at its N terminus that is required for transport to plastids (Figure 8).

Heme oxygenase activity has also been described in algae. In cyanobacteria, red algae, and cryptophytes, phycobilin pigments, which are structurally similar to BV or bilirubin, are attached to biliproteins and function as accessory photosynthetic antenna pigments. The enzymatic properties of algal heme oxygenase are different from those of animals (Troxler et al., 1979). Algal heme oxygenase from *Cyanidium caldarium* has been characterized enzymatically as a soluble and ferredoxin-dependent enzyme localized in plastids (Rhie and Beale, 1992, 1995). In contrast, animal heme oxygenase is a microsomal enzyme for heme catabolism requiring NADPH–cytochrome P450 reductase. The gene product of *HY1* is predicted to be a soluble protein by using Kyte–Doolittle hydropathy analysis based on the amino acid sequence (data not shown). The Arabidopsis HY1 protein does not have a hydrophobic domain for microsomal membrane association at its C terminus, as has been observed in animal heme oxygenase. Instead, the HY1 protein contains a transit peptide that was sufficient for the transport of the GFP to plastids (Figure 8A).

Immunoblot analysis showed that the soluble HY1 protein accumulated in plastids (Figure 8E). These results indicate that the HY1 protein is a soluble plastid protein. This conclusion is in good agreement with the observation that heme oxygenase activity is localized in plastids in algae (Cornejo and Beale, 1988) and that heme oxygenase activity can be detected in isolated plastids (Terry and Kendrick, 1996; Weller et al., 1996). The site of biosynthesis of the phytochrome chromophore likely resides in plastids in higher plants because the ferrochelatase gene encodes a plastid transit peptide (Smith et al., 1994), and the activity for phytochromobilin synthase was shown to be in plastids (Terry and Lagarias, 1991).

Mutants with phenotypes similar to Arabidopsis *hy1* have been identified in other species (Terry, 1997). The pea mutant, *pcd1*, which is unable to convert heme to BV IXα, was useful for providing biochemical evidence that the conversion of heme to BV IXα is an enzymatic process in higher plants (Weller et al., 1996). The tomato mutant *yellow-green-2* was shown to be deficient in BV IXα synthesis (Terry and Kendrick, 1996). Although it is possible that these mutants are related to the regulatory function for heme oxygenase activity, the availability of a clone for Arabidopsis *HY1*

should be useful in identifying the biological deficiencies in these mutants. In fact, both the *pcd1* mutant of pea and the *yellow-green-2* mutant of tomato lack a protein that is recognized by our Arabidopsis anti-HY1 antibody in their respective wild types (M.J. Terry, personal communication).

Long hypocotyl mutants have been isolated extensively in Arabidopsis, and the *hy2* and *hy6* mutants appear phenotypically similar to *hy1*. Although one of the possible explanations is that they have an overlapping function with *HY1*, the heme oxygenases in animals, which show sequence similarity to *HY1*, are not composed of heterogeneous subunits. Although the *hy1*-conferred phenotype is rescued by BV, the *hy2*-conferred phenotype is rescued only partially by BV (Parks and Quail, 1991). Another similar mutation, *hy6*, is clearly different from *hy1* based on genetic analysis (Chory et al., 1989), although currently available *hy6* seeds, distributed from the Arabidopsis Biological Resource Center, have a mutation in the *HY1* gene. Recently, the lesion in the mutant seeds has been renamed *hy1-100* (Terry, 1997). Further molecular characterization of the *HY2* and *HY6* genes is needed to understand their functional relationship with *HY1*.

Although we have cloned the *HY1* gene, this work does not elucidate the mechanism(s) of developmental regulation of phytochrome chromophore biogenesis. The phenotypic effect of *hy1* is much more severe in seedlings, but *hy1* mutants are relatively healthy in the late stages of plant development. One possible explanation for this phenotype is that there is a redundant gene(s) responsible for phytochrome chromophore biosynthesis during the later stages of plant development. If this is true, molecular cloning of the *HY1* gene should help to identify the redundant gene in the Arabidopsis genome. We have in fact identified another gene that shows similarity to *HY1* in the nucleotide sequence database of the Arabidopsis Genome Initiative project (data not shown). We are now investigating whether this gene, which is similar to *HY1*, is functionally involved in phytochrome chromophore biosynthesis.

The results presented here indicate that *HY1* encodes a heme oxygenase required for phytochrome chromophore biosynthesis and also for normal photomorphogenesis. Molecular cloning of *HY1* provides a powerful new tool to analyze biochemically phytochrome chromophore biosynthesis and to analyze developmental regulation of tetrapyrrole biosynthesis in plants.

METHODS

Plant Materials and Libraries

Plants (*Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta*) were from our laboratory stocks. Mutant strains used in this work were obtained from M. Koornneef (Wageningen Agricultural University, Wageningen, The Netherlands) for *hy1*(21.84N), F. Ausubel (Massachusetts General Hospital, Boston, MA) for *hy1 as ER*(807), M. Caboche (INRA, Versailles, France) for *hy1*(E512), and the Arabidopsis Biological Resource Center (Columbus, OH) for *hy1-100*, which was previously distributed as *hy6*(CS236).

Arabidopsis yeast artificial chromosome (YAC) libraries used for chromosome walking were obtained from C. Somerville (Carnegie Institute of Washington, Stanford, CA) for the EG library (Grill and Somerville, 1991) and E. Ward (Novartis, Research Triangle Park, NC) for the EW library (Ward and Jen, 1990). A cDNA library, λPRL2 (CD4-7), was obtained from the Arabidopsis Biological Resource Center. A genomic library from Arabidopsis ecotype Columbia, used for the isolation of the genomic clone for *HY1*, was constructed in λ FixII (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Chromosome Walking

λ restriction fragment length polymorphism (RFLP) clones m220 and m283 were obtained from E. Meyerowitz (California Institute of Technology, Pasadena, CA; Chang et al., 1988), and cosmid clone g6842 was from our laboratory stock (Nam et al., 1989). A DNA fragment to be used as a probe for *GPA1* was synthesized by polymerase chain reaction (PCR) by using primers based on the published sequence (Ma et al., 1990). Yeast colony hybridization was conducted as described (Brownstein et al., 1989). Total DNA from the candidate YAC clones was used to confirm, by DNA gel blot hybridization, the results from the YAC colony hybridization. The end fragments of the YAC insert were recovered by inverse PCR (Ochman et al., 1988). DNA gel blot hybridizations to detect potential RFLPs used Columbia and Landsberg *erecta* DNA digested with six to 30 restriction enzymes.

Codominant Cleaved Amplified Polymorphic Sequence Markers

Two codominant cleaved amplified polymorphic sequence (CAPS) markers between Columbia and Landsberg *erecta* were developed. The CAPS marker *HY1* was designed to detect a single nucleotide substitution at the first exon in the *HY1* gene. Primer sequences 5′-CATTGCGTGACTTCTTCTGTAACCA-3′ and 5′-GATTACCATCCTAAGAGTACTTC-3′ were designed for CAPS marker HY1. The amplified DNA fragment for HY1 is 740 nucleotides in length. It contains a single HpaII site in Landsberg *erecta* but not in Columbia.

The CAPS marker ks450 was located between *HY1* and the right end of the insert in YAC EG12B5-2. Primer sequences 5′-CGGTAGCCGATCCTGATTTGATCAG-3′ and 5′-TTCCTTATCTCCTTGTCTAACTTCC-3′ were designed for ks450. The amplified DNA fragment for ks450 is 620 nucleotides in length. It contains a single HpaII site in Landsberg *erecta* but not in Columbia.

Subcloning of YACs into λ FixII

Two YACs (EW20C4 and EG12B5-2) were isolated using pulse field gel electrophoresis in a 1% low-melting-point agarose gel. YAC DNA in the agarose gel was partially digested with Sau3AI, and the ends were partially filled in by using two nucleotides. Digested DNA was size fractionated on an agarose gel and used for subcloning into λ FixII. A contig of the λ FixII clones was assembled by fingerprinting (Hauge et al., 1991) and hybridization analyses.

Constructs in the Binary Vector pBIN19

The λ FixII vector has two NotI sites flanking the cloning site that can be used to cut out the insert DNA. The binary vector pBIN19 (Bevan, 1984) has a unique NotI site, but not at the cloning site in the T-DNA region. Therefore, pBIN19 was cut at the unique XmaI site in the T-DNA region, and the ends were filled in partially by using two nucleotides with dCTP. The inserts in 20 λ clones were cut out by NotI digestion, filled in by two nucleotides with dGTP at both ends, and ligated to the partially filled-in XmaI site of pBIN19. The resulting plasmids were amplified in *Escherichia coli* and then introduced into *Agrobacterium tumefaciens* LBA4404 and EHA105 by electroporation. The stability of the constructs in Agrobacterium was checked by DNA gel blot hybridization.

Plant Transformation

Transgenic Arabidopsis plants were regenerated from roots or hypocotyl explants of the *hy1*(21.84N) mutant after coculture with Agrobacterium strains harboring binary constructs for overlapping genomic DNA fragments (Valvekens et al., 1988).

Phytochrome Photoreversible Assay in Vitro

Arabidopsis seeds were irradiated with red light, germinated, and grown in the dark for 5 days. Phytochrome difference spectra (Pfr–Pr) were measured for crude extracts from seedlings with a dual-wavelength difference spectrophotometer (model 557; Hitachi Ltd., Tokyo, Japan) (Pratt, 1983).

RNA Gel Blot Hybridization

Arabidopsis RNA was isolated from 9-day-old seedlings according to a published protocol (Ausubel et al., 1987). Hybridization was performed according to a standard method (Church and Gilbert, 1984).

Localization Experiment Using a Green Fluorescent Protein Fusion

The coding sequence of *HY1* for the transit peptide and flanking four amino acid residues (amino acids 1 to 59) was fused to the N terminus of a modified green fluorescent protein (*GFP*) gene under the control of the cauliflower mosaic virus 35S promoter (35SΩ–sGFP[S65T]) (Chiu et al., 1996). The construct was introduced into tobacco leaves by bombardment (Bio-Rad PDS-1000/He apparatus, 1 μg of plasmid; 1.5 mg of 1.6-μm gold particles; chamber vacuum of 28 inches Hg; helium pressure of 1100 psi; 8-cm target distance). Transient expression was observed, after overnight incubation, by using fluorescence microscopy (Axiophoto [Zeiss, Jena, Germany] filter sets; excitation BP450-490; beamsplitter FT510; emission BP515-565).

Protein Extraction and Chloroplast Isolation

Plants were grown on Murashige and Skoog plates (Murashige and Skoog, 1962) at 23°C for 4 days after germination. Seedlings were harvested and frozen by liquid nitrogen. Frozen seedlings (100 mg) were homogenized and extracted using a mortar and pestle with 100 μL of extraction buffer. The extraction buffer consisted of 0.1 M Tris, pH 7.8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.7 μg/mL pepstatin, 1 μg/mL aprotinin, 0.5 μg/mL leupeptin, and 2 mM DTT. The homogenate was separated into soluble and insoluble fractions by centrifugation for 15 min at 14,000*g*.

Rosette leaves were harvested from plants grown for 3 weeks on soil in the greenhouse. Homogenates were separated into top, broken chloroplast, and intact chloroplast fractions on a Percoll step gradient (Cline et al., 1985; Robinson and Barnett, 1988).

Construction of a Plasmid Expressing a Recombinant HY1 Protein

PCR was used to construct a *HY1* cDNA without the region encoding the transit peptide sequence. The reaction mixture contained standard reagents, with *HY1* cDNA as template and synthetic primers (HY1NGEX, 5′-CGGAATTCCGCTACTACTGCGGCAGAGAAGCAG-3′; HY1-R, 5′-GAGATA*TCTAGA*GACACTGCGCACC-3′). The upstream primer HY1NGEX corresponds to nucleotide sequence positions +166 to +189 of the *HY1* cDNA (from initiation codon) and contains an EcoRI linker (underlined sequence). The downstream primer *HY1*-R corresponds to nucleotide sequence positions +853 to +877 of the *HY1* cDNA (from initiation codon) and contains an XbaI site (italic). The amplified DNA was digested with EcoRI and XbaI and cloned into pBluescript II KS+ (Stratagene). After confirmation by nucleotide sequence analysis, the insert fragment was recovered by EcoRI and NotI digestion and cloned into pGEX4T-3 to express the glutathione *S*-transferase (GST)–HY1ΔTP fusion protein.

Preparation of *E. coli* Crude Extract

A fresh single colony of *E. coli* JM109 transformed with the plasmid expressing the GST–HY1ΔTP fusion protein was cultured overnight at 37°C in 3 mL of Luria–Bertani medium. Five hundred microliters of culture incubated overnight was used to inoculate 30 mL of Luria–Bertani medium. The cells were grown at 37°C to mid-log phase, and then GST–HY1ΔTP was induced by adding 1 mM isopropyl *B*-D-thiogalactopyranoside. After incubating for 3 hr, cells were harvested and washed with 10 mL of wash buffer (20 mM Tris, pH 7.8, 20 mM NaCl, and 1 mM EDTA). The cells were then resuspended in 2.5 mL of lysis buffer (50 mM Tris, pH 7.8, 100 mM NaCl, 0.05% Triton X-100, 2 μg/mL leupeptin, 2 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 3 μg/mL pepstatin). The mixture was sonicated for 30 sec on ice. The lysate was centrifuged at 100,000*g* for 30 min, and the supernatant was collected.

For production of the anti-HY1 antibody, GST–HY1ΔTP was purified with a glutathione–Sepharose column, according to the manufacturer's manual (Pharmacia Biotechnology, Uppsala, Sweden). Purified GST–HY1ΔTP was digested with thrombin, and GST was removed according to the manufacturer's manual (Pharmacia Biotechnology). HY1ΔTP was collected and used for production of the anti-HY1 antibody.

Detection of Heme Oxygenase Activity in *E. coli* Crude Extracts

Heme oxygenase activity was determined by measuring bilirubin formation monitored as an absorbance increase at 450 nm, according to Lincoln et al. (1988), Ito-Maki et al. (1995), and Rhie and Beale (1995), with some modifications. The reaction mixture contained, in a

final volume of 1 mL, 0.1 M Tris, pH 7.8, 15 nmol of hemin, 0.15 mg of BSA, recombinant cyanobacteria biliverdin reductase (BVR) (45 nmol bilirubin per hr), 0.25 μmol of NADPH, 50 μg of spinach ferredoxin, 0.025 units of spinach ferredoxin–$NADP^+$ reductase, 2 μmol of Tiron (Dojin, Kumamoto, Japan), and the soluble fraction of the *E. coli* crude extract. The reaction was initiated by the addition of NADPH. The reaction was incubated for 10 min at 25°C. The expression plasmid containing the gene encoding BVR in *Synechocystis* spp PCC6803 was constructed by the J.C. Lagarias laboratory (University of California, Davis). Recombinant cyanobacteria BVR with strep-tag was expressed in *E. coli* and purified with streptavidin-agarose (Skerra, 1994).

Immunochemical Detection of HY1 Protein

Proteins (10 μg) were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. Protein gel blot analysis was performed according to a published protocol by using the anti-HY1 antibody as the primary antibody and horseradish peroxidase–conjugated anti–rabbit IgG antibody as the secondary antibody (Harlow and Lane, 1988). Blots were developed using the enhanced chemiluminescent detection system for protein gel blots (ECL; Amersham, Braunschweig, Germany).

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1987). Current Protocols in Molecular Biology. (New York: Greene Publishing Associates and Wiley-Interscience).

Becker, T.W., Foyer, C., and Caboche, M. (1992). Light-regulated expression of the nitrate reductase and nitrite reductase genes in tomato and in the phytochrome-deficient *aurea* mutant of tomato. Planta **188,** 39–47.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12,** 8711–8721.

Bowler, C., and Chua, N.-H. (1994). Emerging themes of plant signal transduction. Plant Cell **6,** 1529–1541.

Brownstein, B.H., Silverman, G.A., Little, R.D., Burke, D.T., Korsmeyer, S.J., Schlessinger, D., and Olson, M.V. (1989). Isolation of single-copy human genes from a library of yeast artificial chromosome clones. Science **244,** 1348–1351.

Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S., and Meyerowitz, E.M. (1988). Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **85,** 6856–6860.

Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996). Engineered GFP as a vital reporter in plants. Curr. Biol. **6,** 325–330.

Chory, J., Peto, C.A., Ashbaugh, M., Saganich, R., Pratt, L., and Ausubel, F. (1989). Different roles for phytochrome in etiolated and green plants deduced from characterization of *Arabidopsis thaliana* mutants. Plant Cell **1,** 867–880.

Chory, J., Chatterjee, M., Cook, R.K., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A., Poole, D., Reed, J., and Vitart, V. (1996). From seed germination to flowering, light controls plant development via the pigment phytochrome. Proc. Natl. Acad. Sci. USA **93,** 12066–12071.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Clack, T., Mathews, S., and Sharrock, R.A. (1994). The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: The sequences and expression of *PHYD* and *PHYE.* Plant Mol. Biol. **25,** 413–427.

Cline, K., Werner-Washburne, M., Lubben, T.H., and Keegstra, K. (1985). Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. J. Biol. Chem. **260,** 3691–3696.

Cornejo, J., and Beale, S.I. (1988). Algal heme oxygenase from *Cyanidium caldarium.* J. Biol. Chem. **263,** 11915–11921.

Cornejo, J., Beale, S.I., Terry, M.J., and Lagarias, J.C. (1992). Phytochrome assembly, the structure and biological activity of 2(*R*),3(*E*)-phytochromobilin derived from phycobiliproteins. J. Biol. Chem. **267,** 14790–14798.

Elich, T.D., and Lagarias, J.C. (1989). Formation of a photoreversible phycocyanobilin-apophytochrome adduct *in vitro.* J. Biol. Chem. **264,** 12902–12908.

Gavel, Y., and von Heijne, G. (1990). A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett. **261,** 455–458.

Grill, E., and Somerville, C. (1991). Construction and characterization of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. Mol. Gen. Genet. **226,** 484–490.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Hauge, B.M., Giraudat, J., Hanley, S., Hwang, I., Kohchi, T., and Goodman, H.M. (1991). Physical mapping of the *Arabidopsis* genome and its applications. In Plant Molecular Biology 2,

R.G. Herrmann and B. Larkins, eds (New York: Plenum Press), pp. 239–248.

Ito-Maki, M., Ishikawa, K., Matera, K.M., Sato, M., Ikeda-Saito, M., and Yoshida, T. (1995). Demonstration that histidine 25, but not 132, is the axial heme ligand in rat heme oxygenase-1. Arch. Biochem. Biophys. **317,** 253–258.

Kendrick, R.E., and Kronenberg, G.H.M., eds (1994). Photomorphogenesis in Plants. (Dordrecht, The Netherlands: Kluwer Academic Publishers).

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403–410.

Koornneef, M., Rolff, E., and Spruit, C.J.P. (1980). Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* L. Heynh. Z. Pflanzenphysiol. **100,** 147–160.

Lagarias, J.C., and Lagarias, D.M. (1989). Self-assembly of synthetic phytochrome holoprotein *in vitro.* Proc. Natl. Acad. Sci. USA **86,** 5778–5780.

Lagarias, J.C., and Rapoport, H. (1980). Chromopeptide from phytochrome. The structure and linkage of the Pr form of the phytochrome chromophore. J. Am. Chem. Soc. **102,** 4821–4828.

Lassner, M.W., Lardizabal, K., and Metz, J.G. (1996). A jojoba β-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell **8,** 281–292.

Lincoln, B.C., Mayer, A., and Bonkovsky, H.L. (1988). Microassay of heme oxygenase by high-performance liquid chromatography: Application to assay of needle biopsies of human liver. Anal. Biochem. **170,** 485–490.

Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1990). Molecular cloning and characterization of *GPA1*, a G protein α subunit gene from *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **87,** 3821–3825.

Matera, K.M., Zhou, H., Migita, C.T., Hobert, S.E., Ishikawa, K., Katakura, K., Maeshima, H., Yoshida, T., and Ikeda-Saito, M. (1997). Histidine-132 does not stabilize a distal water ligand and is not an important residue for the enzyme activity in heme oxygenase-1. Biochemistry **36,** 4909–4915.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant. **15,** 473–497.

Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol. **102,** 269–277.

Nam, H.-G., Giraudat, J., den Boer, B., Moonan, F., Loos, W.D.B., Hauge, B.M., and Goodman, H.M. (1989). Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana.* Plant Cell **1,** 699–705.

Ochman, H., Gerber, A.S., and Hartl, D.L. (1988). Genetic appli–cations of an inverse polymerase chain reaction. Genetics **120,** 621–623.

Oelmüller, R., and Kendrick, R.E. (1991). Blue light is required for survival of the tomato phytochrome-deficient *aurea* and the expression of four nuclear genes for plastidic proteins. Plant Mol. Biol. **16,** 293–299.

Parks, B.M., and Quail, P.H. (1991). Phytochrome-deficient *hy*1 and *hy*2 long hypocotyl mutants of Arabidopsis are defective in phytochrome chromophore biosynthesis. Plant Cell **3,** 1177–1186.

Pratt, L.H. (1983). Assay of photomorphogenic photoreceptors. In Photomorphogenesis, W.J. Shropshire and H. Mohr, eds (Berlin: Springer-Verlag), pp. 152–177.

Quail, P.H. (1991). Phytochrome: A light activated molecular switch that regulates plant gene expression. Annu. Rev. Genet. **25,** 389–409.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M., and Chory, J. (1994). Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol. **104,** 1139–1149.

Rhie, G., and Beale, S.I. (1992). Biosynthesis of phycobilins: Ferredoxin-supported NADPH-independent heme oxygenase and phycobilin-forming activities from *Cyanidium caldarium.* J. Biol. Chem. **267,** 16088–16093.

Rhie, G., and Beale, S.I. (1995). Phycobilin biosynthesis: Reductant requirements and product identification for heme oxygenase from *Cyanidium caldarium.* Arch. Biochem. Biophys. **320,** 182–194.

Robinson, C., and Barnett, L.K. (1988). Isolation and characterization of chloroplasts. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford: IRL Press), pp. 67–78.

Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.M., Gaymard, F., and Grignon, C. (1992). Cloning and expression in yeast of a plant potassium ion transport system. Science **256,** 663–665.

Sharrock, R.A., and Quail, P.H. (1989). Novel phytochrome sequences in *Arabidopsis thaliana*: Structure, evolution and differential expression of a plant regulatory photoreceptor family. Genes Dev. **3,** 1745–1757.

Shinomura, T., Nagatani, A., Chory, J., and Furuya, M. (1994). The induction of seed germination in *Arabidopsis thaliana* is regulated principally by phytochrome B and secondarily by phytochrome A. Plant Physiol. **104,** 363–371.

Shinomura, T., Nagatani, A., Hanzawa, H., Kubota, M., Watanabe, M., and Furuya, M. (1996). Action spectra for phytochrome A- and B-specific photoinduction of seed germination in *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **93,** 8129–8133.

Skerra, A. (1994). A general vector, pASK84, for cloning bacterial production, and single-step purification of antibody F-Ab fragments. Gene **141,** 79-84.

Smith, A.G., Santana, M.A., Wallace-Cook, A.D.M., Roper, J.M., and Labbe-Bois, R. (1994). Isolation of a cDNA encoding chloroplast ferrochelatase from *Arabidopsis thaliana* by functional complementation of a yeast mutant. J. Biol. Chem. **269,** 13405–13413.

Smith, H. (1995). Physiological and ecological function within the phytochrome family. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 289–315.

Sun, J., and Loehr, T.M. (1994). Identification of histidine 25 as the heme ligand in human liver heme oxygenase. Biochemistry **33,** 13734–13740.

Suzuki, T., Sato, M., Ishikawa, K., and Yoshida, T. (1992). Nucleotide sequence of cDNA for porcine heme oxygenase and its expression in *Escherichia coli.* Biochem. Int. **28,** 887–893.

Takahashi, S., Wang, J., Rousseau, D., Ishikawa, K., Yoshida, T., Host, J.R., and Ikeda-Saito, M. (1994a). Heme–heme oxygenase complex: structure of the catalytic site and its implication for oxygen activation. J. Biol. Chem. **269,** 1010–1014.

Takahashi, S., Wang, J., Rousseau, D., Ishikawa, K., Yoshida, T., Takeuchi, N., and Ikeda-Saito, M. (1994b). Heme–heme oxygen-

ase complex: Structure and properties of the catalytic site from resonance raman scattering. Biochemistry **33,** 5531–5538.

Takahashi, S., Ishikawa, K., Takeuchi, N., Ikeda-Saito, M., Yoshida, T., and Rousseau, D. (1995). Oxygen-bound heme–heme oxygenase complex: Evidence for a highly bent structure of the coordinated oxygen. J. Am. Chem. Soc. **117,** 6002–6006.

Terry, M.J. (1997). Phytochrome chromophore-deficient mutants. Plant Cell Environ. **20,** 740–745.

Terry, M.J., and Kendrick, R.E. (1996). The *aurea* and *yellow-green-2* mutants of tomato are deficient in phytochrome chromophore synthesis. J. Biol. Chem. **271,** 21681–21686.

Terry, M.J., and Lagarias, J.C. (1991). Holophytochrome assembly. Coupled assay for phytochromobilin synthase *in organello.* J. Biol. Chem. **266,** 22215–22221.

Terry, M.J., Wahleithner, J.A., and Lagarias, J.C. (1993). Biosynthesis of the plant photoreceptor phytochrome. Arch. Biochem. Biophys. **306,** 1–15.

Terry, M.J., McDowell, M.T., and Lagarias, J.C. (1995). (3*Z*)- and (3*E*)-Phytochromobilin are intermediates in the biosynthesis of the phytochrome chromophore. J. Biol. Chem. **270,** 11111–11118.

Troxler, R.F., Brown, A.S., and Brown, S.B. (1979). Bile pigment synthesis in plants. J. Biol. Chem. **254,** 3411–3418.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis thaliana* root explants using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

van Tuinen, A., Hanhart, C.J., Kerckhoffs, L.H.J., Nagatani, A., Boylan, M.T., Quail, P.H., Kendrick, R.E., and Koornneef, M. (1996). Analysis of phytochrome-deficient *yellow-green-2* and *aurea* mutants of tomato. Plant J. **9,** 173–182.

von Heijne, G., Steppuhn, J., and Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180,** 535–545.

Ward, E.R., and Jen, G.C. (1990). Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly-sheared *Arabidopsis thaliana* DNA. Plant Mol. Biol. **14,** 561–568.

Weller, J.L., Terry, M.J., Rameau, C., Reid, J.B., and Kendrick, R.E. (1996). The phytochrome-deficient *pcd1* mutant of pea is unable to convert heme to biliverdin IXα. Plant Cell **8,** 55–67.

Weller, J.L., Terry, M.J., Reid, J.B., and Kendrick, R.E. (1997). The phytochrome-deficient *pcd2* mutant of pea is unable to convert biliverdin IXα to 3(*Z*)-phytochromobilin. Plant J. **11,** 1177–1186.

Yoshida, T., Biro, P., Cohen, T., Muller, R.M., and Shibahara, S. (1988). Human heme oxygenase cDNA and induction of its mRNA by hemin. Eur. J. Biochem. **171,** 457–461.

Yoshida, T., Ishikawa, K., and Sato, M. (1991). Degradation of heme by a soluble peptide of heme oxygenase obtained from rat liver microsomes by mild trypsinization. Eur. J. Biochem. **199,** 729–733.