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

Takuya Muramoto, Takayuki Kohchi, Akiho Yokota, Inhwan Hwang, and Howard M. Goodman

Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan
Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

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(FR)-sensing phytochrome family, blue(UV-A) photoreceptors termed cryptochromes, and UV-B photoreceptors (Kendrick and Kronenberg, 1994). Of these, 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 (PFB) 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 PFB 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)-PFB and subsequently isomerized to PFB (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...
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). 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, hy1er and hy1as. These double mutant lines in the Landsberg background were crossed to wild-type Columbia plants. The F1 progeny were, as expected, phenotypically wild type, because all of the mutations are recessive. Those F2 progeny having a recombinational breakpoint in the vicinity of HY1 were collected, and the breakpoints were mapped using F3 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 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
Cloning of Arabidopsis HY1

vector pBIN19, and the T-DNA was introduced into the hy1 mutant by Agrobacterium-mediated transformation (Valvekens et al., 1988). The regenerants (T₁ 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 T₂ 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 T₃ 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 photoreversible 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>
<thead>
<tr>
<th>Molecular Marker</th>
<th>Enzyme Used for RFLP Analysis</th>
<th>Number of Recombinants</th>
<th>Total Number of Plants Examined</th>
<th>Relative Distances ( ^{a} )</th>
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</table>

\(^{a}\) 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.
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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 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) pro-

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.
Cloning of Arabidopsis HY1

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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 4. Difference Spectra of Crude Extracts from Etiolated Seedlings.

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

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 microsomal 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 trans 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 serine). Furthermore, the consensus sequence of the cleavage site, (Val/Ile)_{1}X_{2}(Ala/Cys)_{1}--Ala_{1} (Gavel and von Heijne, 1990), was also observed as Val-Val-Ala-Ala at amino acid

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**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 trans 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 (λmax of heme) decreased with time as the absorbance at 450 nm (λmax 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.

**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
Bacco leaves. The heme oxygenase gene encoding the HY1 protein that is similar to animal be due to gene silencing. Therefore, Arabidopsis flowering (T. Muramoto, unpublished observation). This may typic 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, phycoobilin 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 (Rie 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 hydrophy designation 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 (Terry and Kendrick, 1996). Although it is possible that these mutants 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.

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 this clone. One of the genes shows significant sequence similarity to heme oxygenase. The corresponding gene in all this clone. One of the genes shows significant sequence similarity to heme oxygenase. The corresponding gene in all this clone. One of the genes shows significant sequence similarity to heme oxygenase. The corresponding gene in all this clone. One of the genes shows significant sequence similarity to heme oxygenase. The corresponding gene in all
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-conferring phenotype is rescued by BV, the hy2-conferring 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(E512), 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(C5236).

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**

A 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’-CATTGCGTACCTCCTGTAACCC-3’ and 5’-GTTACCATCTC-AAAGGATCTTT-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 EG1285-2. Primer sequences 5’-CGGT-AGCCGATCTCCTGATTTGAC-3’ and 5’-TTCCCTATCTCTTGT-TCTAACTTCC-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 EG1285-2) 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 \( \chi \) Fix1I vector has two Nott sites flanking the cloning site that can be used to cut out the insert DNA. The binary vector pBIN19 (Bevan, 1984) has a unique Ntot 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 2019 have 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 by using 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(2.184N) 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 (35S-\( \Omega \)-GFP[S65T]) (Chiu et al., 1996). The construct was introduced into tobacco leaves by bombardment (Bio-Rad PDS-1000/He apparatus, 1 \( \mu \)g of plasmid; 1.5 mg of 1.6-\( \mu \)-m 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 (Axiphoto [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\( \mu \)L of extraction buffer. The extraction buffer consisted of 0.1 M Tris, pH 7.8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.7 \( \mu \)g/mL peptatin, 1 \( \mu \)g/mL aprotinin, 0.5 \( \mu \)g/mL leupeptin, and 2 mM DTT. The homogenate was separated into soluble and insoluble fractions by centrifugation for 15 min at 14,000g.

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 HY1-NGEX, 5'-CGGATCCGCTACTACTGGCCAGAAGACG-3'; HY1-R, 5'-GATATCTAGAGCATGCGCACC-3'). The upstream primer HY1-NGEX 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 XbaI and cloned into pGEX4T-3 to express the gluthathione S-transferase (GST)-HY1\( \Delta \)TP fusion protein.

Preparation of E. coli Crude Extract

A fresh single colony of E. coli J M109 transformed with the plasmid expressing the GST–HY1\( \Delta \)TP 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–HY1\( \Delta \)TP was induced by adding 1 mM isopropyl \( \beta \)-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 \( \mu \)g/mL leupeptin, 2 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 3 \( \mu \)g/mL peptatin). The mixture was sonicated for 30 sec on ice. The lysate was centrifuged at 100,000g for 30 min, and the supernatant was collected.

For production of the anti-HY1 antibody, GST–HY1\( \Delta \)TP was purified with a glutathione–Sepharose column, according to the manufacturer’s manual (Pharmacia Biotechnology, Uppsala, Sweden). Purified GST–HY1\( \Delta \)TP was digested with thrombin, and GST was removed according to the manufacturer’s manual (Pharmacia Biotechnology). HY1\( \Delta \)TP 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), Tso-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).

**Immunoochemical 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).

**ACKNOWLEDGMENTS**

We thank the Arabidopsis Biological Resource Center (Columbus, OH), Drs. Maarten Koornneef (Wageningen Agricultural University, Wageningen, The Netherlands), Frederick Ausubel (Massachusetts General Hospital, Boston, MA), Chris Somerville (Carnegie Institute of Washington, Stanford, CA), Michel Caboche (INRA, Versailles, France), Elliot Meyerowitz (California Institute of Technology, Pasadena, CA), Eric Ward (Novartis, Research Triangle Park, NC), and Yasuo Niwa and Hirokazu Kobayashi (University of Shizuoka, Shizuoka, Japan) for providing us with clones, DNA, and seed. We also thank Dr. Satoru Tokutomi (University of Osaka Prefecture, Sakai, Japan) for helping to detect heme oxygenase activity of recombinant HY1 protein and for providing a strain. We are grateful to Kenji Ohaya (Kyoto University, Kyoto, Japan) for providing us with a grant-in-Aid from the Ministry of Education, Science, Sport, and Culture, Japan, to T.K.

Received October 9, 1998; accepted January 25, 1999.

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