Plant δ-Aminolevulinic Acid Dehydratase

Expression in Soybean Root Nodules and Evidence for a Bacterial Lineage of the Alad Gene

Christine M. Kaczor, Michael W. Smith, Indu Sangwan, and Mark R. O'Brian*

Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214 (C.M.K., I.S., M.R.O'B.); and Molecular Genetics Laboratory and Human Genome Center, The Salk Institute, San Diego, California 92186 (M.W.S).

We isolated a soybean (Glycine max) cDNA encoding the heme and chlorophyll synthesis enzyme δ-aminolevulinic acid (ALA) dehydratase by functional complementation of an Escherichia coli hemB mutant, and we designated the gene Alad. ALA dehydratase was strongly expressed in nodules but not in uninoculated roots, although Alad mRNA was only 2- to 3-fold greater in the symbiotic tissue. Light was not essential for expression of Alad in leaves of dark-grown etiolated plantlets as discerned by mRNA, protein, and enzyme activity levels; hence, its expression in subterranean nodules was not unique in that regard. The data show that soybean can metabolize the ALA it synthesizes in nodules, which argues in favor of tetrapyrrole formation by the plant host in that organ. Molecular phylogenetic analysis of ALA dehydratases from 11 organisms indicated that plant and bacterial enzymes have a common lineage not shared by animals and yeast. We suggest that plant ALA dehydratase is descended from the bacterial endosymbiont ancestor of chloroplasts and that the Alad gene was transferred to the nucleus during plant evolution.

Plants synthesize a wide variety of tetrapyrroles that include Chl, hemes, siroheme, and bilins, and these compounds participate in many cellular processes (reviewed by Beale and Weinstein, 1991). Synthesis of hemes and Chl from the universal tetrapyrrole precursor ALA share common enzymic steps and diverge after protoporphyrin IX synthesis for formation of the respective metal porphyrin derivative. Chl is by far the predominant tetrapyrrole found in most plants, and its abundance in photosynthetic tissues has rendered it amenable to experimental analysis. Accordingly, much less is known about the expression of other tetrapyrroles that are amenable to experimental analysis. Accordingly, much less is known about the expression of other tetrapyrroles that are functionally important but are usually found in low amounts.

One exception is plant hemoglobin expressed in legume root nodules, which makes up approximately 25% of the plant protein mass in the cytosol (Appleby, 1984). Nodules are specialized root organs elicited by nitrogen-fixing rhizobia (reviewed by Nap and Bisseling, 1990; Caetano-Anolles and Gresshoff, 1991; Verma, 1992; Long and Staskawicz, 1993), and hemoglobin is one of numerous nodule-specific plant proteins essential for symbiosis. Despite the unique context in which plant hemoglobin is expressed, aspects of heme precursor synthesis in soybean (Glycine max) nodules have features in common with tetrapyrrole formation described in leaves. Plants synthesize ALA from glutamate in leaves by the C₅ pathway (reviewed by Beale and Weinstein, 1991; Jahn et al., 1992), and the C₅ pathway enzyme GSA aminotransferase is expressed in root nodules (Sangwan and O'Brian, 1993). ALA formation activity is activated in nodules (Sangwan and O'Brian, 1991, 1992), and this is at least in part due to induction of the GSA aminotransferase gene (Gsa) (Sangwan and O'Brian, 1993). The soybean Gsa gene is strongly expressed in leaves of etiolated plantlets and is reduced in older leaves; thus Gsa is regulated in leaves as well.

Synthesis of the leghemoglobin heme prosthetic group has been a topic of controversy, and it underscores the lack of information about tetrapyrrole synthesis by the plant host in nodules. The hypothesis that nodule hemoglobin heme is a bacterial product has been put forth (Cutting and Schulman, 1969), and that idea is based primarily on the lack of heme synthesis enzyme activities in plant fractions of nodules (Cutting and Schulman, 1969; Porra, 1975; Nadler and Avissar, 1977) and on the leghemoglobin-defective phenotype of nodules induced by some rhizobial heme synthesis mutants (Leong et al., 1982; O'Brian et al., 1987). The former argument is undermined somewhat by the discovery of a soybean nodule ALA formation activity (Sangwan and O'Brian, 1991, 1992, 1993), and the analysis of mutants must be viewed in the context of data that show that nodules elicited by a Rhizobium meliloti hemA mutant are arrested in development at a stage that precedes hemoglobin expression (Dickstein et al., 1991). Similarly, Bradyrhizobium japonicum mutants defective in hemB (Chauhan and O'Brian, 1993) and hemH (Frustaci and O'Brian, 1992, 1993) incite soybean nodules that are poorly developed; thus, the absence of late-nodule proteins such as hemoglobin is expected regardless of the source of prosthetic group. Thus, the symbiont that carries out hemoglobin heme synthesis remains unknown.

Evidence suggests that Bradyrhizobium japonicum can take up and utilize soybean-derived ALA (Sangwan and O'Brian, 1991). Synthesis of the leghemoglobin heme prosthetic group has been a topic of controversy, and it underscores the lack of information about tetrapyrrole synthesis by the plant host in nodules. The hypothesis that nodule hemoglobin heme is a bacterial product has been put forth (Cutting and Schulman, 1969), and that idea is based primarily on the lack of heme synthesis enzyme activities in plant fractions of nodules (Cutting and Schulman, 1969; Porra, 1975; Nadler and Avissar, 1977) and on the leghemoglobin-defective phenotype of nodules induced by some rhizobial heme synthesis mutants (Leong et al., 1982; O'Brian et al., 1987). The former argument is undermined somewhat by the discovery of a soybean nodule ALA formation activity (Sangwan and O'Brian, 1991, 1992, 1993), and the analysis of mutants must be viewed in the context of data that show that nodules elicited by a Rhizobium meliloti hemA mutant are arrested in development at a stage that precedes hemoglobin expression (Dickstein et al., 1991). Similarly, Bradyrhizobium japonicum mutants defective in hemB (Chauhan and O'Brian, 1993) and hemH (Frustaci and O'Brian, 1992, 1993) incite soybean nodules that are poorly developed; thus, the absence of late-nodule proteins such as hemoglobin is expected regardless of the source of prosthetic group. Thus, the symbiont that carries out hemoglobin heme synthesis remains unknown.

Evidence suggests that Bradyrhizobium japonicum can take up and utilize soybean-derived ALA (Sangwan and O'Brian, 1991). Synthesis of the leghemoglobin heme prosthetic group has been a topic of controversy, and it underscores the lack of information about tetrapyrrole synthesis by the plant host in nodules. The hypothesis that nodule hemoglobin heme is a bacterial product has been put forth (Cutting and Schulman, 1969), and that idea is based primarily on the lack of heme synthesis enzyme activities in plant fractions of nodules (Cutting and Schulman, 1969; Porra, 1975; Nadler and Avissar, 1977) and on the leghemoglobin-defective phenotype of nodules induced by some rhizobial heme synthesis mutants (Leong et al., 1982; O'Brian et al., 1987). The former argument is undermined somewhat by the discovery of a soybean nodule ALA formation activity (Sangwan and O'Brian, 1991, 1992, 1993), and the analysis of mutants must be viewed in the context of data that show that nodules elicited by a Rhizobium meliloti hemA mutant are arrested in development at a stage that precedes hemoglobin expression (Dickstein et al., 1991). Similarly, Bradyrhizobium japonicum mutants defective in hemB (Chauhan and O'Brian, 1993) and hemH (Frustaci and O'Brian, 1992, 1993) incite soybean nodules that are poorly developed; thus, the absence of late-nodule proteins such as hemoglobin is expected regardless of the source of prosthetic group. Thus, the symbiont that carries out hemoglobin heme synthesis remains unknown.

Evidence suggests that Bradyrhizobium japonicum can take up and utilize soybean-derived ALA (Sangwan and O'Brian, 1991).

1 This work was supported by National Science Foundation grant IBN-9204778 and by the Cooperative State Research Service, U.S. Department of Agriculture, under agreement 91-37305-6750.

* Corresponding author; fax 1-716-829-2725.

Abbreviations: ALA, δ-aminolevulinic acid; GSA, glutamate 1-semialdehyde; PBG, porphobilinogen.
1991), thereby rendering the bacterial hemA gene nonessential for that symbiosis (Guerinot and Chelmi, 1986). Therefore, plant ALA is not committed to plant tetrapyrrole synthesis, and induction of ALA formation activity cannot in itself be interpreted as evidence for plant heme synthesis in nodules. Very recently, we found that, unlike the hemA gene, the B. japonicum hemB gene is essential for symbiosis (Chauhan and O’Brien, 1993), suggesting that ALA is the only plant tetrapyrrole precursor that can be utilized by the bacterial symbiont. It follows then that soybean nodule enzymes involved in ALA metabolism should be committed to plant tetrapyrrole synthesis; so we have initiated a study of the expression of soybean ALA dehydratase, the enzyme that forms PBG from ALA. In addition, we provide molecular phylogenetic evidence for a bacterial origin for plant ALA dehydratases, indicating that the Alad gene was derived from the bacterial ancestor of chloroplasts and was transferred to the nucleus during plant evolution.

**MATERIALS AND METHODS**

Plants and Bacteria

*Escherichia coli* strain RP523 is a hemB mutant of strain C600 and is a hemin auxotroph (10 µg/mL) (Li et al., 1988). *E. coli* strains were grown in Luria broth, and ampicillin (50 µg/mL) was added to media for growth of strains harboring pUC18, pSK Bluescript II, and their recombinant derivatives. *Bradyrhizobium japonicum* strain II10 was the soybean endosymbiont used in the present work and was grown in GSY media as previously described (Frustaci et al., 1991). Soybeans (*Glycine max* cv Essex), either inoculated with *B. japonicum* or uninoculated, were grown from 23-d-old plants for enzyme assays or RNA extraction.

**Isolation and Analysis of Soybean Leaf cDNA Encoding ALA Dehydratase**

A soybean leaf cDNA expression vector library in pUC18 was a gift from Dr. M.L. Kahn (Washington State University, Pullman, WA) and was constructed as described by Udvardi et al. (1991). Library DNA was transformed to the *E. coli* hemB mutant strain RP523 en masse by electroporation, and cells were subsequently plated onto Luria broth (Ausubel et al., 1987) containing isopropyl β-D-thiogalactopyranoside (0.4 mM) and ampicillin in the absence of hemin. Complemented transformants were scored as ampicillin-resistant hemin prototrophs. Plasmid DNA was isolated from the transformed cells as described previously (Ausubel et al., 1987) and analyzed initially by agarose gel electrophoresis and retransformation of strain RP523 to confirm that the plasmid conferred hemin prototrophy on the mutant. The cDNA insert of one of the complementing clones, pTK1, was cloned into pBluescript IISK+ to construct pRRK1, and deletions were made using an Exo-Mung deletion kit (Stratagene). The nucleotide sequence of both strands of the pRRK1 insert was determined by the chain termination method (Sanger et al., 1977) using a Sequenase kit (United States Biochemical) according to the manufacturer’s instructions.

**ALA Dehydratase Protein and Enzyme Activity**

The enzyme activity of ALA dehydratase was measured as PBG formation from ALA as described previously (Sangwan and O’Brien, 1991). Extracts of *E. coli* and of soybean leaves, roots, and nodule cytosol were prepared as described previously (Sangwan and O’Brien, 1993). The absence of bacterial contamination in plant nodule fractions is observed as the lack of ALA synthase activity in the plant fractions (Sangwan and O’Brien, 1991). Reactions were carried out for 1 h at 37°C in Tricine buffer (pH 8), 8 mM ALA, and 20 mM DTT using 0.5 mg of protein per reaction. Control reactions were incubated on ice. PBG was quantitated spectrophotometrically after reaction with the Ehrlich reagent as described by Sangwan and O’Brien (1992). ALA dehydratase was detected in plant tissue extracts immunologically using antibodies raised against the spinach enzyme (Schaumburg et al., 1992) in slot blots. Cross-reactive protein on nitrocellulose filters was discerned visually using peroxidase-conjugated goat anti-rabbit IgG as described previously (Ausubel et al., 1987).

**RNA Isolation and Analysis**

Nodules, leaves, and roots were excised, frozen in liquid nitrogen, and homogenized in a blender with homogenization buffer and phenol (2:2:3, w/v/v). The homogenization buffer contained 500 mM Tris (pH 8), 10 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 0.5% (w/v) deoxycholate, and 1 mM β-mercaptoethanol. Total RNA was isolated from the homogenate as described previously (Ausubel et al., 1987), and poly(A)+ RNA was isolated using oligo(dT)-cellulose columns. Northern blot analysis of poly(A)+ RNA was carried out as previously described under high-stringency conditions (Ausubel et al., 1987). Ubiquitin cDNA was provided by Dr. D.P.S. Verma (Fortin et al., 1988). Relative amounts of RNA detected in northern blots were estimated by scanning densitometry of the autoradiograms.

**Analysis of ALA Dehydratase Sequences and Construction of Phylogenetic Trees**

Deduced amino acid sequences of 11 ALA dehydratases were trimmed to the conserved regions and progressively aligned with the TREE program of Feng and Doolittle (1990), which uses the Dayhoff Mutation Matrix (Dayhoff, 1978). Matrix distances are calculated from the number of shared residues in pairwise comparisons, their randomized sequences, and self-comparisons of each (Feng and Doolittle, 1990). The ALA dehydratase sequences analyzed in the present work are from soybean, pea (Boese et al., 1991), spinach and *Selaginella martensii* (Schaumburg et al., 1992), human (Wetmur et al., 1986), mouse (Bishop et al., 1989), Saccharomyces cerevisiae (yeast, Myers et al., 1987), *E. coli* (Li et al., 1989), Bacillus subtilis (Hansson et al., 1991), *B. japonicum* (Chauhan and O’Brien, 1993), and Methanothermus sociabilis (Brock et al., 1992). The rat ALA dehydratase (Bishop et al., 1986) was not included in the analysis because of its near identity to the mouse sequence.
Isolation of aALA Dehydratase-Encoding cDNA

*E. coli* strain RP523 is defective in hemB, the gene encoding ALA dehydratase, and behaves as a hemin auxotroph (Li et al., 1988). Strain RP523 was transformed on masse with a soybean leaf cDNA expression vector library, and cells that were functionally complemented were screened as ampicillin-resistant hemin prototroph colonies on agar media. Fifteen prototrophic transformants were isolated that bore identical plasmids as judged from restriction digest patterns, and one prototrophic transformant was isolated that behaved as a hemin auxotroph (Li et al., 1991). This identity, along with the complementation of the *E. coli* hemB mutant, provides very strong evidence that the cloned cDNA encodes soybean ALA dehydratase, and the corresponding gene is designated *Alad*. The cDNA sequence shows that *Alad* mRNA is polyadenylated at the 3' end (Fig. 1), indicating that the RNA was processed in the nucleus. Because ALA dehydratases are plastid-localized proteins (Smith, 1988), it must be targeted to that organelle from the cytoplasm, and the amino-terminal features that typify such domains (Gavel and von Heijne, 1990).

Expression of the Alad Gene in Soybean Tissues

Poly(A)^+ RNA from leaves, nodules, and uninfected (asymmetrically) roots of 23-d-old plants was probed with *Alad* cDNA in northern blots to discern the enzyme in soybean tissues; cross-reactive protein was found in leaves and nodules but not in uninfected roots (Fig. 3). ALA dehydratase enzyme activities in the respective tissues were consistent with the antibody data, showing activity in leaves and nodules but no discernible activity in uninfected roots (Fig. 3). It is unlikely that the ALA dehydratase found in nodule cytosol was due to *B. japonicum* contamination during fractionation because bacterial extracts expressed only one-fourth of the activity of that found in the plant cytosol (4 versus 16 nmol h^-1 mg^-1 protein). In addition, ALA synthase activity, a bacterial marker enzyme, is absent in the plant fraction of nodules. These observations indicate that *Alad* gene expression in nodules is controlled primarily at the synthesis or turnover of the enzyme and not of the mRNA; Boese et al. (1991) reached a similar conclusion for control of ALA dehydratase in pea leaves. Although we

### Table 1. Functional complementation of *E. coli* hemB mutant strain RP523 with soybean cDNA clone pTK1

<table>
<thead>
<tr>
<th>Strain</th>
<th>ALAD Activity</th>
<th>Doubling Time of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmmol PBC h^-1 mg^-1 protein</td>
<td>min</td>
</tr>
<tr>
<td>C600[pUC18]</td>
<td>11.2</td>
<td>39</td>
</tr>
<tr>
<td>RP523[pUC18]</td>
<td>0</td>
<td>No growth</td>
</tr>
<tr>
<td>RP523[pTK1]</td>
<td>6.6</td>
<td>43</td>
</tr>
</tbody>
</table>

RESULTS

Expression of the *Alad* Gene in Soybean Tissues

Poly(A)^+ RNA from leaves, nodules, and uninfected (asymmetrically) roots of 23-d-old plants was probed with *Alad* cDNA in northern blots to discern *Alad* transcripts in those tissues (Fig. 2). *Alad* mRNA was expressed in root nodules as discerned by a single band, and the steady-state level was 2- to 3-fold greater than was found in uninfected roots (Fig. 2). The modest increase of *Alad* message in nodules compared to roots differs from that of *Gsa*, which is much greater in the symbiotic tissue than in uninfected tissues (Sangwan and O' Brian, 1993; Fig. 2). The single band at 1.6 kb in all tissues examined suggested that the message represents a single gene or gene family. Antibodies raised against spinach ALA dehydratase (Schaumburg et al., 1992) were used to detect the enzyme in soybean tissues; cross-reactive protein was found in leaves and nodules but not in uninfected roots (Fig. 3). ALA dehydratase enzyme activities in the respective tissues were consistent with the antibody data, showing activity in leaves and nodules but no discernible activity in uninfected roots (Fig. 3). It is unlikely that the ALA dehydratase found in nodule cytosol was due to *B. japonicum* contamination during fractionation because bacterial extracts expressed only one-fourth of the activity of that found in the plant cytosol (4 versus 16 nmol h^-1 mg^-1 protein). In addition, ALA synthase activity, a bacterial marker enzyme, is absent in the plant fraction of nodules. These observations indicate that *Alad* gene expression in nodules is controlled primarily at the synthesis or turnover of the enzyme and not of the mRNA; Boese et al. (1991) reached a similar conclusion for control of ALA dehydratase in pea leaves. Although we

Figure 1. Nucleotide sequence and deduced product of the cloned cDNA insert of pTK1 encoding soybean ALA dehydratase. The underlined region represents the putative leader peptide.

Copyright © 1994 American Society of Plant Biologists. All rights reserved.

---

**Table 1. Functional complementation of *E. coli* hemB mutant strain RP523 with soybean cDNA clone pTK1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ALAD Activity</th>
<th>Doubling Time of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmmol PBC h^-1 mg^-1 protein</td>
<td>min</td>
</tr>
<tr>
<td>C600[pUC18]</td>
<td>11.2</td>
<td>39</td>
</tr>
<tr>
<td>RP523[pUC18]</td>
<td>0</td>
<td>No growth</td>
</tr>
<tr>
<td>RP523[pTK1]</td>
<td>6.6</td>
<td>43</td>
</tr>
</tbody>
</table>

**RESULTS**

**Isolation of a ALA Dehydratase-Encoding cDNA**

*E. coli* strain RP523 is defective in hemB, the gene encoding ALA dehydratase, and behaves as a hemin auxotroph (Li et al., 1988). Strain RP523 was transformed on masse with a soybean leaf cDNA expression vector library, and cells that were functionally complemented were screened as ampicillin-resistant hemin prototroph colonies on agar media. Fifteen prototrophic transformants were isolated that bore identical plasmids as judged from restriction digest patterns, and one of them, pTK1, was chosen for subsequent experiments. pTK1 conferred on strain RP523 hemin prototrophy and ALA dehydratase activity, which was approximately one-half of that found in parent strain C600 (Table I). The insert of pTK1 contained an open reading frame of 412 amino acids beginning with a Met codon (Fig. 1), and the deduced protein shared 88% identity with the ALA dehydratase from pea (Boese et al., 1991). This identity, along with the complementation of the *E. coli* hemB mutant, provides very strong evidence that the cloned cDNA encodes soybean ALA dehydratase, and the corresponding gene is designated *Alad*. The cDNA sequence shows that *Alad* mRNA is polyadenylated at the 3' end (Fig. 1), indicating that the RNA was processed in the nucleus. Because ALA dehydratases are plastid-localized proteins (Smith, 1988), it must be targeted to that organelle from the cytoplasm, and the amino-terminal features that typify such domains (Gavel and von Heijne, 1990).

**Expression of the Alad Gene in Soybean Tissues**

Poly(A)^+ RNA from leaves, nodules, and uninfected (asymmetrically) roots of 23-d-old plants was probed with *Alad* cDNA in northern blots to discern *Alad* transcripts in those tissues (Fig. 2). *Alad* mRNA was expressed in root nodules as discerned by a single band, and the steady-state level was 2- to 3-fold greater than was found in uninfected roots (Fig. 2). The modest increase of *Alad* message in nodules compared to roots differs from that of *Gsa*, which is much greater in the symbiotic tissue than in uninfected tissues (Sangwan and O'Brian, 1993; Fig. 2). The single band at 1.6 kb in all tissues examined suggested that the message represents a single gene or gene family. Antibodies raised against spinach ALA dehydratase (Schaumburg et al., 1992) were used to detect the enzyme in soybean tissues; cross-reactive protein was found in leaves and nodules but not in uninfected roots (Fig. 3). ALA dehydratase enzyme activities in the respective tissues were consistent with the antibody data, showing activity in leaves and nodules but no discernible activity in uninfected roots (Fig. 3). It is unlikely that the ALA dehydratase found in nodule cytosol was due to *B. japonicum* contamination during fractionation because bacterial extracts expressed only one-fourth of the activity of that found in the plant cytosol (4 versus 16 nmol h^-1 mg^-1 protein). In addition, ALA synthase activity, a bacterial marker enzyme, is absent in the plant fraction of nodules. These observations indicate that *Alad* gene expression in nodules is controlled primarily at the synthesis or turnover of the enzyme and not of the mRNA; Boese et al. (1991) reached a similar conclusion for control of ALA dehydratase in pea leaves. Although we

Figure 1. Nucleotide sequence and deduced product of the cloned cDNA insert of pTK1 encoding soybean ALA dehydratase. The underlined region represents the putative leader peptide.
whether light was necessary for expression of the Alad gene in leaves. Poly(A)* RNA was isolated from leaves of etiolated soybean plants grown completely in darkness and from greening etiolated plant leaves made so by exposure to light for 24 h prior to harvesting. The soybean cab gene was used as a control for a light-induced gene (Chang and Walling, 1992), and it can be seen in northern blots that expression of that gene is strongly dependent on light (Fig. 4). However, Alad mRNA levels did not require light for high expression, as seen by the pronounced hybridization signal in RNA from leaves of dark-grown plantlets. The Alad message was enhanced only 2-fold by light, which is similar to what we previously found for the Gsa gene (Sangwan and O’Brien, 1993; Fig. 4). Because leaves of etiolated plants expand considerably during the 24-h greening period, it is plausible that the modest differences in Alad and Gsa messages expressed in the respective tissues reflect developmental control of those genes. The protein and enzyme activity of ALA dehydratase were expressed in leaves of dark-grown plants at levels at least as high as was found in light-exposed leaves (Fig. 3). From this, we conclude that full expression of ALA dehydratase in leaves does not require light.

Evidence for a Bacterial Lineage for Plant ALA Dehydratases

The putative metal-binding domain of the B. japonicum ALA dehydratase contains some features characteristic of the corresponding domain in the plant enzymes (Chauhan and O’Brien, 1993). In the present work, we wanted to determine whether the anomalous B. japonicum protein could be the result of an evolutionary horizontal gene transfer from the plant host to the bacterium, in which case the B. japonicum

![Image: Northern blot analysis poly(A)* RNA from leaves (L), roots (R), and nodules (N) of 23-d-old plants. RNA (5 µg) was loaded onto each lane, and the figure represents a composite of a single filter probed separately with cDNA encoding ALA dehydratase (Alad), GSA aminotransferase (Gsa), or ubiquitin (Ubi). Ubiquitin was used as a constitutively expressed gene.](image)

![Image: Immunoblot and enzyme activities of ALA dehydratase from soybean tissue extracts. Protein (25 µg per slot) was loaded and probed with antibodies raised against spinach ALA dehydratase. The enzyme activity of ALA dehydratase was carried out as described in the text, and the data are expressed as nmol PBG formed h−1 mg−1 protein and are averages of three trials. Tissue extracts were prepared from leaves (L), roots (R), and nodules (N) of 23-d-old plants and from the leaves of etiolated plantlets that were either subjected to complete darkness (D) or illuminated for 24 h prior to harvesting (I), in which time they greened.](image)

![Image: Effect of light on Alad mRNA levels in leaves of etiolated plantlets. Poly(A)* RNA was isolated from leaves of plantlets grown completely in darkness (D) or illuminated for 24 h prior to harvesting (I), in which time they greened. RNA (5 µg) was loaded onto each lane, and the figure represents a composite of a single filter hybridized separately with radiolabeled DNA probes. The cDNA probes used encode ALA dehydratase (Alad), Chl a/b-binding protein (Cab), GSA aminotransferase (Gsa), and ubiquitin (Ubi). Cab and Ubi were used as controls for light-induced and constitutive genes, respectively.](image)
ALA dehydratase would be unique in its similarity to the plant enzymes over its entire length. Conserved regions of 11 ALA dehydratases from plants, bacteria, yeast, and mammals were progressively aligned as described by Feng and Doolittle (1990), and phylogenetic trees were constructed (Fig. 5). The analysis as depicted in the unrooted matrix tree did not indicate a plant source for the B. japonicum ALA dehydratase, but rather it infers a bacterial origin of plant ALA dehydratases. The sequence similarities between each of the plant-bacteria comparisons (50–58%) were higher than any of those between plants and other eukaryotes (39–47%). Grouping of the archaeabacterium M. sociabilis with the eu-
bacteria is an unexplained anomaly of the phylogenetic
analysis (Fig. 5). The endosymbiotic theory of organelle orig-
ination proposes that chloroplasts (and mitochondria) are
derived from once free-living bacteria (Gray, 1989). Thus,
the deduced phylogeny of ALA dehydratases, along with the
known plastid location of the nuclear-encoded enzyme, leads
us to postulate that the plant Alad gene is descended from
the bacterial ancestor of chloroplasts and was transferred to
the nucleus during plant evolution.

**DISCUSSION**

In the present work, we isolated a cDNA encoding soybean
ALA dehydratase and examined the expression of the Alad

![Figure 5. Molecular phylogenetic analysis of ALA dehydratase from various organisms.](image)

Figure 5. Molecular phylogenetic analysis of ALA dehydratase from various organisms. Deduced amino acid sequences of 11 ALA dehydratases were trimmed to the conserved regions and pro-
gressively aligned with the TREE program of Feng and Doolittle (1990),
which uses the Dayhoff Mutation Matrix (Dayhoff, 1978). The ALA
dehydratase sequences analyzed in the present work are from
soybean (Fig. 2), pea (Boese et al., 1991), spinach and S. martensii
(Schaumburg et al., 1992), human (Wetmur et al., 1986), mouse
(Bishop et al., 1989), S. cerevisiae (yeast, Myers et al., 1987), E. coli
(Li et al., 1989), B. subtilis (Hansson et al., 1991), B. japonicum
(Chauhan and O’Brien, 1993), and M. sociabilis (Brockl et al., 1992).
The rat ALA dehydratase (Bishop et al., 1986) was not included
in the analysis because of its near identity to the mouse sequence.

gene in root nodules as a step toward assessing whether the
plant host can synthesize significant quantities of heme in
that organ. Soybean ALA dehydratase was highly expressed
in nodules but not in uninfected roots, and thus, the Alad
gene was induced by symbiosis. Alad mRNA was only mod-
estly higher in nodules than in uninfected roots; hence, the
large differences in enzyme expression between the two
tissues are likely to be due primarily to protein synthesis or
stability. This level of control is unlike that of the GSA
aminotransferase gene in nodules, in which the transcript
level contributes significantly to differential expression (Sang-
wan and O’Brien, 1993). Although Chl synthesis requires
light, expression of Alad did not require light in leaves of
etiolated plantlets; thus, there is no need to invoke a regula-
tory factor to compensate for light to account for the expres-
sion of ALA dehydratase in subtained root nodules. Our
studies with etiolated plants do not rule out light regulation
of the Alad gene in leaves of plants grown under a normal
diurnal light/dark cycle.

The expression of ALA dehydratase by soybean in nodules
strongly suggests that plant-synthesized Alad can be metab-
ilized by the host as well as by the bacterial symbiont. This
conclusion is significant because plant PBG cannot support
B. japonicum heme formation in nodules (Chauhan and
O’Brien, 1993); hence, the soybean ALA dehydratase is com-
mitted to plant tetrapyrrole synthesis. Expressions of soybean
Alad and Gsa are comparable to those found in leaves in
which copious amounts of Chl are synthesized (Figs. 2 and
3; Sangwan and O’Brien, 1993); thus, it is clear that the
activities of those enzymes are sufficient to support heme
formation in high quantities. A diminution of the induced
ALA formation and ALA dehydratase activities as a function
of nodule age (Nadler and Avissar, 1977; Sangwan and
O’Brien, 1992) infers that rates of synthesis are highest in
young nitrogen-fixing nodules. The data do not prove that
hemoglobin heme is provided, in part or in toto, by the
eukaryotic symbiont, but it alters the premises on which past
arguments have been made (Cutting and Schulman, 1969).

In addition, we reiterate that soybean nodules elicited by
B. japonicum hemB or hemH mutants are underdeveloped (Frus-
taci and O’Brien, 1992; Chauhan and O’Brien, 1993); thus,
the lack of leghemoglobin, a late-nodule protein, is
guessed regardless of the source of the prosthetic group.

Molecular phylogenetic analysis suggests that plant and
bacterial ALA dehydratases have a common lineage not
shared by animals or yeast; we interpret this as evidence that
plant ALA dehydratase is derived from the bacterial chloro-
plast ancestor and was transferred to the nucleus during plant evolution. Paleobotanical and molecular evidence iden-
tifies the lycopsids, the group of primitive tracheophytes to
which Selaginella belongs, as the basal lineage in vascular
land plants (Raubeson and Jansen, 1992, and refs. therein).
Thus, if one assumes that the nuclear location of the bacteria-
type Alad genes analyzed herein is the result of a single
transfer, the event would have occurred no less than 400
million years ago, and the nuclear-encoded gene should be
prevalent in vascular land plants. Transfer of the Alad gene
from the chloroplast to the nucleus may be considerably
more ancient, and the identification and localization of the
gene from nonvascular plants (bryophytes) and algae should clarify this.

Separate origins for ALA dehydratase between plants and animals indicates that the Chl pathway in plants is not likely the result of an evolutionary addition of a unique branch to the heme pathway enzymes of the ancestor from which plants and animals diverged approximately 1 billion years ago. Also, the absence of a cytosolic ALA dehydratase in plants (Smith, 1988) suggests that the eukaryotic type Alad gene predicted to be inherited from that ancestor has been lost or inactivated. Evidence suggests that Chl-specific plant enzymes are descended from the chloroplast ancestor (Fujita et al., 1992; Bauer et al., 1993; Burke et al., 1993), and the present work points to the same origin for a universal tetrapyrole synthesis enzyme.

NOTE ADDED IN PROOF

A soybean gene encoding coproporphyrinogen oxidase, which catalyzes the antepenultimate step in heme synthesis, is highly expressed in root nodules (Madsen et al., 1993). This observation supports the conclusion that the plant host can form heme in nodules.

ACKNOWLEDGMENTS

The authors thank Sharon Cosloy for E. coli strain RP523, Michael Kahn for the soybean leaf cDNA library, H.A.W. Schneider-Poetsch for anti-spinach ALA dehydratase antibodies, Desh Pal Verma for the ubiquitin cDNA, and Linda Walling for the soybean leaf cDNA library, H.A.W. Schneider-Poetsch for the ubiquitin cDNA, and Linda Walling for cab3 cDNA.

Received October 8, 1993; accepted December 13, 1993.

Copyright Clearance Center: 0032-0889/94/104/1411/07.

LITERATURE CITED


Bishop TR, Frelin LP, Boyer SH (1986) Nucleotide sequence of rat liver 6-aminolevulinic acid dehydratase cDNA. Nucleic Acids Res 14: 1015


Fortin MG, Purohit SK, Verma DPS (1988) The primary structure of soybean (Glycine max) is identical to other plant ubiquitins. Nucleic Acids Res 16: 11377


Guerinot ML, Chelnik BK (1986) Bacterial 6-aminolevulinic acid synthase is not essential for leghemoglobin formation in the soybean Bradyrhizobium japonicum symbiosis. Proc Natl Acad Sci USA 83: 1837-1841


Expression and Lineage of Plant δ-Aminolevulinic Acid Dehydratase


O’Brian MR, Kirshbom PM, Maier RJ (1987) Bacterial heme synthesis is required for expression of the leghemoglobin holoprotein but not the apoprotein in soybean root nodule. Proc Natl Acad Sci USA 84: 8390–8393


Schaumburg A, Schneider-Poetsch HAW, Eckerskorn C (1992) Characterization of plastid δ-aminolevulinate dehydratase (ALAD; EC4.2.1.24) from spinach (Spinacia oleracea L.) by sequencing and comparison with non-plant ALAD enzymes. Z Naturforsch 47c: 77–84


