Molecular Characterization and Subcellular Localization of Protoporphyrinogen Oxidase in Spinach Chloroplasts[1]

Fang-Sik Che[2]*, Naohide Watanabe[2], Megumi Iwano, Hachiro Inokuchi, Seiji Takayama, Shigeo Yoshida, and Akira Isogai

Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916–5, Takayama Ikoma, Nara 630–0101, Japan (F.S.C., N.W., M.I., S.T., A.I.); Department of Biophysics, Faculty of Science, Kyoto University, Sakyoku, Kyoto, 606–8502, Japan (H.I.); and The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2–1, Wako-shi, Saitama, 351–0198, Japan (S.Y.)

Protoporphyrinogen oxidase (Protox) is the last common enzyme in the biosynthesis of chlorophylls and heme. In plants, there are two isoenzymes of Protox, one located in plastids and other in the mitochondria. We cloned the cDNA of spinach (*Spinacia oleracea*) plastidal Protox and purified plastidal Protox protein from spinach chloroplasts. Sequence analysis of the cDNA indicated that the plastid Protox of spinach is composed of 562 amino acids containing the glycine-rich motif GxGxxG previously proposed to be a dinucleotide binding site of many flavin-containing proteins. The cDNA of plastidal Protox complemented a Protox mutation in *Escherichia coli.* N-terminal sequence analysis of the purified enzyme revealed that the plastidal Protox precursor is processed at the N-terminal site of serine-49. The predicted transit peptide (methionine-1 to cysteine-48) was sufficient for the transport of precursors into the plastid because green fluorescent protein fused with the predicted transit peptide was transported to the chloroplast. Immunocytochemical analysis using electron microscopy showed that plastidal Protox is preferentially associated with the stromal side of the thylakoid membrane, and a small portion of the enzyme is located on the stromal side of the chloroplast inner envelope membrane.

Tetrapyrrole biosynthesis is important in plants because it provides to many essential molecules involved in light harvesting, energy transfer, signal transduction, detoxification, and systemic acquired resistance (von Wettstein et al., 1995; Grimm, 1998; Molina et al., 1999). The most abundant tetrapyrroles are chlorophyll and heme, which are important compounds for photosynthesis and respiration.

Protoporphyrinogen oxidase (Protox, EC 1.3.3.4) is the last enzyme in the common pathway of chlorophyll and heme biosynthesis (Beale and Weinstein, 1990). Protox catalyzes the oxidative O_2-dependent aromatization of the colorless protoporphyrinogen IX (Protogen IX) to the highly conjugated protoporphyrin IX (Proto IX). Protox is also the target enzyme of phthalimide-type herbicides such as S23142 (*N*-[4-chloro-2-fluoro-5-propagyloxy]-phenyl-3,4,5,6-tetrahydrophthalimide) and diphenylether-type herbicides such as acifluorfen (AF; 5-[2-chloro-4-(trifluoromethyl) phenoxy]-2-nitrobenzoic acid) (Sato et al., 1987; Matringe et al., 1989, 1992a; Witkowski and Halling, 1989; Scalla et al., 1990; Varsano et al., 1990; Camadro et al., 1991; Duke et al., 1991; Jacobs et al.,

1991). Protox has been reported to be widely distributed among plants, animals, and bacteria (Dailey, 1990; Camadro et al., 1999). Studies of the structure and function of Protox have been stimulated by the discovery that herbicides are very potent inhibitors of the Protox activities of yeast, mammal, and plant mitochondria and plant plastids in vitro (Matringe et al., 1992b; Che et al., 1993; Lee et al., 1993; Lee and Duke, 1994; Arnould et al., 1997; Birchfield et al., 1998). In mammals and yeast, Protox activity was detected in mitochondrial inner membrane (Dailey, 1990), while in plants, Protox activity has been observed in both plastids and mitochondria (Jacobs and Jacobs, 1987, 1993; Matringe et al., 1992b; Smith et al., 1993). The genes of Protox were first identified from *Escherichia coli* (Sasarman et al., 1993) and *Bacillus subtilis* (Hansson and Hederstedt, 1994) and have been designated *hemG* and *hemY*. The predicted M_rs of HemG and HemY are different, and there is no sequence similarity between them. These enzymes represent two distinct Protogen-oxidizing systems: the HemY-type oxygen-dependent system and the bacterial multicomponent system.

In plants, the characteristics of chloroplast and mitochondrial Protox enzymes have long been controversial, since two different cDNAs of tobacco (*Nicotiana tabacum* cv Samsun NN) have been identified by complementation of the heme auxotrophic *E. coli hemG* mutant lacking Protox activity (Lermontova et al., 1997). One cDNA encodes a protein of 548 amino

Che et al.

acid residues (PPX-I), and the other a protein of 504 amino acid residues (PPX-II). The deduced amino acid sequences of PPX-I and PPX-II have only 27.3% conserved amino acid residues. Because the translation product of PPX-I cDNA could be translocated to plastids, and the translation product of PPX-II cDNA was targeted to mitochondria, PPX-I and PPX-II were termed as plastidal Protox and mitochondrial Protox, respectively (Lermontova et al., 1997). The 53-kD mature protein of plastidal Protox was detected in chloroplasts, suggesting that processing at a plastidal target sequence is needed to translocate into chloroplasts. Although much research has been done, the detailed mechanism of the transport of plastidal Protox is still uncertain.

We recently studied the molecular mechanism of herbicide resistance in tobacco cells (YZI-1S) that had been selected as an S23142-resistant line (Ichinose et al., 1995; Watanabe et al., 1998). Our data indicated that the primary target of the herbicide is plastidal Protox and its inhibition causes serious damage to chloroplast function in wild-type cells (Watanabe et al., 1998). In spinach (*Spinacia oleracea*) the activity of plastidal Protox has been detected entirely in thylakoid membranes and chloroplast envelopes (Matringe et al., 1992b). Protox-inhibiting herbicides such as S23142 and AF inhibited activity in envelope fractions as strongly as in thylakoid fractions. In addition, the binding constants of [^{3}H]AF were similar for envelope membranes and thylakoids (Matringe et al., 1992b). From these data it is likely that the plastidal Protoxes of envelope membranes and thylakoids are the same and not isoenzymes.

Accurate examination of the transport and subcellular localization of chlorophyll and heme biosynthetic enzymes is important for a better understanding of the flux of tetrapyrrole precursors and the regulation of the synthesis of tetrapyrrole. This is particularly important for Protox because it is the last common enzyme of these two pathways (von Wettstein et al., 1995). The aim of this study is to obtain detailed information about plastidal Protox, including the transit sequence and processing sites, and to determine the precise location of plastidal Protox in chloroplasts. We report here the first cloning of spinach plastidal Protox cDNA and the analysis of the N-terminal sequence in mature plastidal Protox purified from spinach chloroplasts and discuss the mechanism of the transport of plastidal Protox into chloroplasts. In addition, we report the precise location of plastidal Protox by western-blot analysis and immuno-electron microscopy.

RESULTS

Isolation and Characterization of Plastidal Protox cDNA from Spinach

To identify cDNAs encoding plastidal Protox in spinach, one set of specific oligonucleotide primers

(SCP-1F and SCP-2R) was synthesized based on conserved sequences in Arabidopsis and tobacco (Narita et al., 1996; Lermontova et al., 1997). PCR amplification of cDNA from spinach produced a product of 936 bp. The nucleotide sequence of the cDNA clone had high homology with the plastidal Protox of Arabidopsis (82%) and tobacco (75%), suggesting that this clone encodes part of the plastidal Protox cDNA of spinach. The full-length plastidal Protox cDNA, *SO-POX1* (accession no. AB029492), was obtained by 5'- and 3'-RACE PCR. Sequence analysis indicated that the plastid Protox of spinach is composed of 562 amino acids with a calculated molecular mass of 59,929 D (Fig. 1). The deduced amino acid sequence of its cDNA shows a very high identity to other plastidal Protoxes (Arabidopsis, 78%; tobacco, 71%; potato, 72%; Fig. 1), while the sequence identities to mitochondrial Protoxes of plants and other organisms (human, yeast, and *Bacillus*) are relatively low (20%–30%) (Hansson and Hederstedt, 1994; Nishimura et al., 1995b; Camadro and Labbe, 1996). In particular, the Gly-rich motif GxGxxG that had been
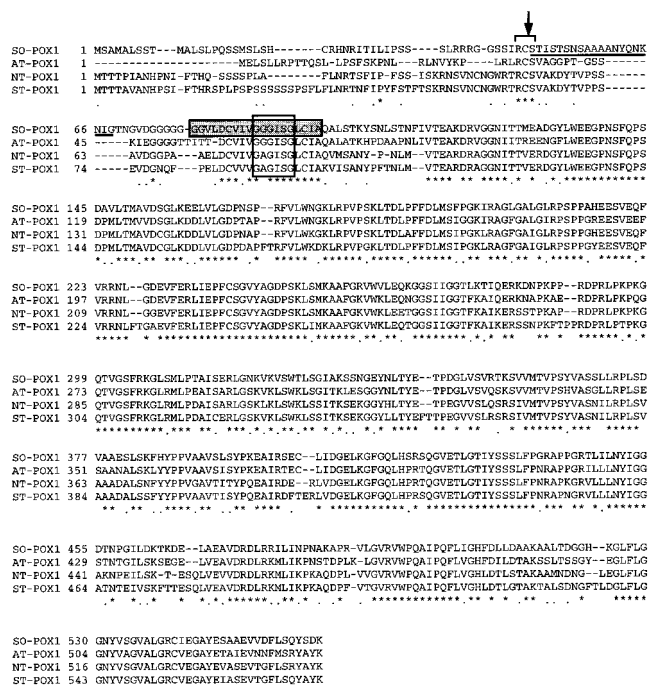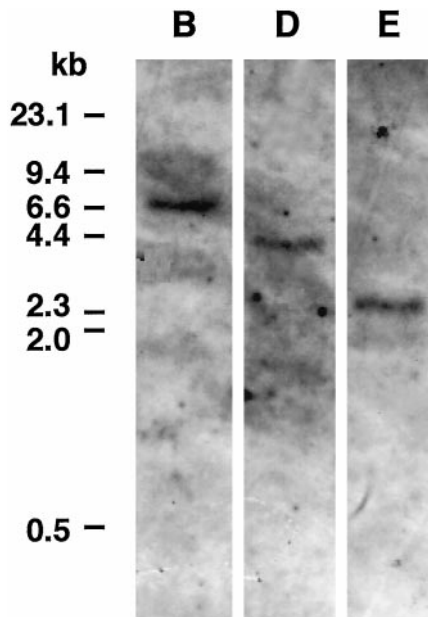

Figure 1. Alignment of the predicted amino acid sequences of plastidal Protox from higher plants. SO, Spinach (this work, AB029492); AT, Arabidopsis (Narita et al., 1996; D83139); NT, tobacco (Lermontova et al., 1997; Y13465); ST, potato (Johnston et al., 1998; AJ225107). Identical amino acid residues for all sequences are indicated by asterisks, and well-conserved sequences are indicated by a dot. The underlined sequence of 20 amino acids was determined by N-terminal sequencing of purified plastidal Protox. The processing site of the transit peptide of spinach plastidal Protox is indicated by an arrow. The three amino acids, Arg-Cys-Ser, at the transit peptide cleavage site are conserved in all known plastid Protoxs. The gray box shows the transmembrane domain predicted by PSORT software and the white box shows the GxGxxG motif.

60

Plant Physiol. Vol. 124, 2000

previously proposed as a dinucleotide binding site of many flavin-containing proteins (Wierenga et al., 1986; Bach et al., 1988; Lermontova et al., 1997; Dailey and Dailey, 1998) was also found in the sequence of spinach plastidal Protox (Fig. 1). Furthermore, the amino acid sequences from position 1 to 60 of spinach plastidal Protox were enriched for Ala, Arg, Gly, Leu, Ser, and Thr but included no Asp or Glu. Since these features have been found in several transit peptides for the transport to plastids (von Heijne et al., 1989; Cline and Henry, 1996), the existence of a transit peptide in the N terminus of plastidal Protox is expected.
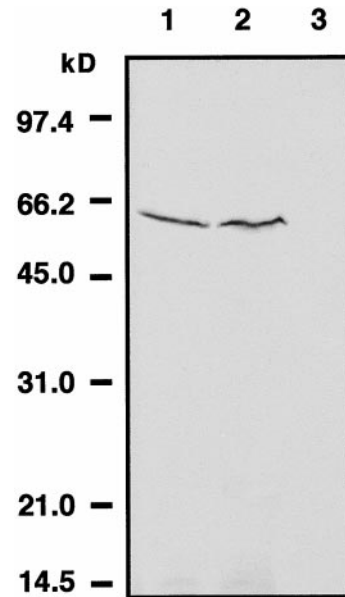
To determine the copy number of the plastidal Protox gene in spinach we performed Southern-blot analysis by digesting spinach genome DNA with *Bgl*II, *Dra*I, and *Eco*RI, which do not cut within the cDNA of plastidal Protox, and hybridized with a labeled DNA probe of the full length of the plastidal Protox cDNA. Hybridization patterns showed a single band in each lane, indicating that there is a single copy of the plastidal Protox gene in the spinach genome (Fig. 2).

Purification and Determination of the N-Terminal Amino Acid Sequence of Plastidal Protox

For characterization of mature plastidal Protox, localization of plastidal Protox was first examined by immunoblot using specific anti-plastidal Protox anti-
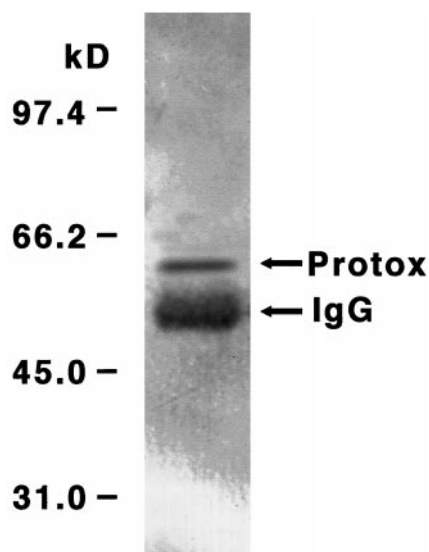

Figure 3. Immunoblot analysis of plastidal Protox in spinach leaf. Proteins (10 μg) from the leaf extract fraction (lane 1), purified chloroplast fraction (lane 2), and mitochondrial fraction (lane 3) were separated on a 12.5% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection was performed with specific antibody raised against the C-terminal region of the recombinant plastidal Protox of spinach. The position of molecular mass markers (in kD) is given at left.

Figure 2. Genomic Southern-blot analysis of plastidal Protox in spinach. Ten micrograms of spinach genomic DNA was digested with restriction enzymes (B, *Bgl*II; D, *Dra*I; and E, *Eco*RI) and subjected to Southern-blot analysis. Hybridization was performed using full-length cDNA of spinach plastidal Protox labeled non-radioactively by random priming. The sizes (in kb) of standard DNA fragments obtained by digesting λ-DNA with *Hind*III are shown at left.

body. A single band with a calculated molecular mass of approximately 60 kD was detected in leaf extracts and chloroplasts (Fig. 3, lanes 1 and 2). In contrast, no signal was detected in the mitochondrial fraction (Fig. 3, lane 3). These results suggest that the plastidal Protox is located only in the chloroplast.

To determine the precise cleavage site in the plastidal Protox precursor, we purified mature plastidal Protox protein from crude extracts by immunoprecipitation. Immunoprecipitation with specific antibodies against the plastidal Protox protein provided a single band except immunoglobulin with Coomassie Brilliant Blue staining (Fig. 4) and the N-terminal sequence analysis of this single band provided the 20-amino acid sequence STISTSNSAAAANYQNK-NIG. From this sequence, the N terminus of mature plastidal Protox was determined to be Ser-49 of plastidal Protox precursor (Fig. 1). This result suggests that plastidal Protox precursor is processed between Cys-48 and Ser-49. This is the first information about the cleavage site of the plastidal Protox precursor. The N-terminal domain (Met-1 to Cys-48) of the plastidal Protox precursor would contain the functional transit peptide sequence for targeting to chloroplasts.

It was reported that cDNAs encoding plastidal Protox of Arabidopsis (Narita et al., 1996) and of tobacco (Lermontova et al., 1997) were able to complement the *hem*G mutation in *E. coli*. The BT3 (Δ*hem*G::Km^r) of *E. coli*, which is defective in the *hem*G gene, grows very poorly even in rich media. Full-length and

Figure 4. SDS-PAGE analysis of plastidal Protox purified by immunoprecipitation and determination of the N-terminal sequence of the purified Protox from spinach chloroplasts. The protein was transferred to a polyvinylidene difluoride membrane and stained with Coomassie Brilliant Blue. The band of plastidal Protox was cut out and directly applied to a protein sequencer. The position of molecular mass markers (in kD) is given at left. The N-terminal sequence of purified protein is shown in the lower panel.

mature-type plastidal Protox cDNA were ligated into vector pCR 2.1 in-frame with *LacZ*. As a control experiment, *hemG* was ligated and introduced in the same manner. Mature plastidal Protox cDNA and *hemG* complements the mutation responsible for the poor growth (data not shown). The full-length cDNA also rescued the mutant cells but to a lesser extent (data not shown). The growth of both complemented strains was inhibited by Protox-inhibiting herbicides such as S23142 and AF at the submicromolar level (data not shown). The transformant introduced in the pCR 2.1 vector without any insert showed poor growth and formed a very small colony (data not shown). These data indicate that the mature-type cDNA of plastidal Protox in spinach can functionally complement the BT3 ($\Delta hemG$::Km^r).

Transport into Chloroplasts

The sequence analysis of plastidal Protox cDNA isolated from spinach and the determination of the N-terminal sequence of the mature protein purified from chloroplasts showed that the N-terminal domain contains a possible transit peptide for targeting to plastids (Figs. 1 and 4). We used the reporter gene green fluorescent protein (GFP) to test if the putative transit peptide is functional. cDNA encoding the predicted transit peptide (Met-1 to Cys-48) and cDNA

encoding the transit peptide plus partial mature protein (Met-1 to Asp-73) were fused with the *GFP2* gene and each fusion placed under the control of cauliflower mosaic virus 35S promoter (M48C-GFP and M73D-GFP). These constructs were introduced into spinach leaves by bombardment, and transient expression was observed by fluorescence microscopy. Coincidence of green fluorescence with chloroplasts was observed in cells bombarded with either transit peptide constructs (Fig. 5, A–D). In the spinach leaf bombarded with control GFP the green fluorescence was spread out over the guard cells (Fig. 5E), indicating that the transit peptide (Met-1 to Cys-48) of spinach plastidal Protox is functional and sufficient to transport proteins to plastids.
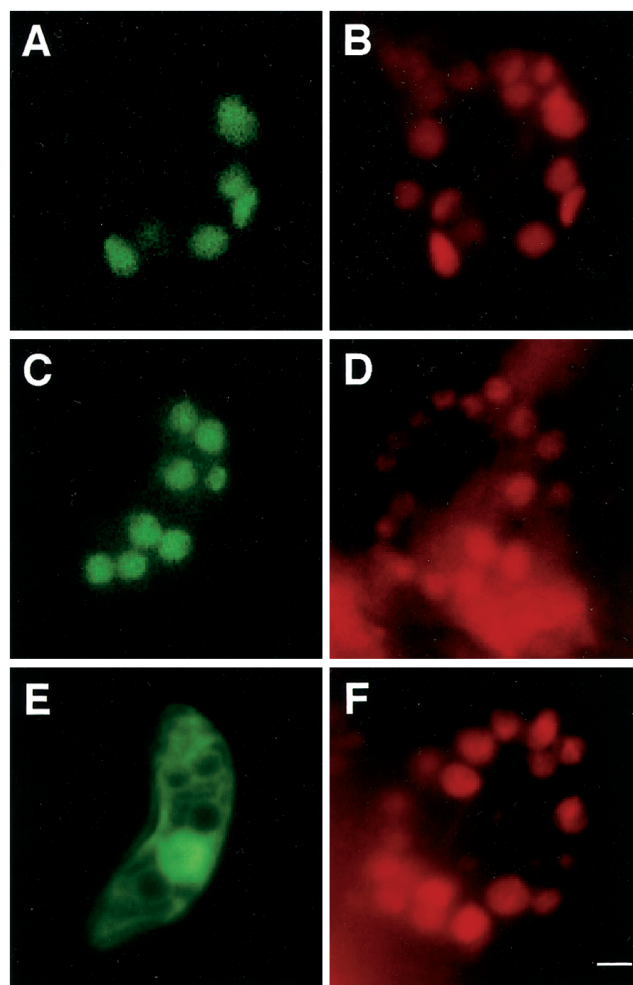
Figure 5. Transport of GFP fused to the N-terminal peptide of spinach plastidal Protox. GFP fused to the N-terminal peptide of spinach plastidal Protox (A and B, M48C-GFP; C and D, M73D-GFP) and GFP (E and F). The fluorescence of GFP was observed at excitation wavelength of 495 nm and emission wavelength of 530 nm (A, C, and E). The autofluorescence of chloroplasts was observed at excitation wavelength of 540 nm and emission wavelength of 600 nm (B, D, and F). Bar = 10 μm.

Organellar Location of Plastidal Protox

Since the plastidal Protox was only located in chloroplasts (Fig. 3), the location of mature plastidal Protox was further examined by immunoblot analysis using specific anti-plastidal Protox antibody. A plastidal Protox band of 60 kD was detected in envelope and thylakoid membrane fractions but not in the stromal fraction (Fig. 6, lanes 2, 3, and 5), suggesting that the plastidal Protox was associated with envelope and thylakoid membranes of chloroplasts. It seems that the mobilities of the Protox band in the envelope membrane fraction was slightly different from the thylakoid membrane fraction on the nitrocellulose membrane (Fig. 6, lanes 2 and 3). However, only one band was detected when the envelope and the thylakoid membrane fractions were loaded on the same lane, suggesting that the molecular mass of the thylakoid Protox was same as that of the envelope Protox.

To identify the spatial distribution of plastidal Protox in chloroplasts we performed an immunogold electron microscopic analysis. When ultra thin sections of leaf tissues from spinach were incubated with antibody against plastidal Protox and with gold-conjugated antiserum to rabbit immunoglobulins, gold particles were found in chloroplasts but not in mitochondria, cytoplasm, vacuoles, or plasma membrane (Fig. 7A). In chloroplasts most of the gold particles were on the stromal side of the thylakoid membrane, and a small number were present on the internal side of the inner envelope membrane (Fig. 7, B and C).

When preimmune serum was used there was no appreciable binding of gold particles (Fig. 7D). In addition, all other control tests, including the omission of primary antibody and pre-incubation of the primary antibody with the plastidal Protox before section labeling, yielded negative results (data not shown). These results clearly show that plastidal Protox is preferentially associated with the stromal side
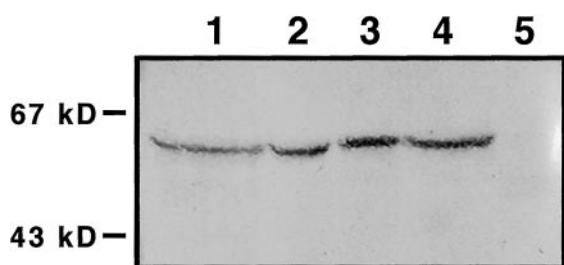

Figure 6. Localization of plastidal Protox within chloroplast of spinach leaf. Proteins from the purified-chloroplast fraction (25 μg, lane 1), thylakoid fraction (10 μg, lane 2), envelope fraction (10 μg, lane 3), and mixed envelope and thylakoid fraction (5 μg each, lane 4) were stroma (10 μg, lane 5) separated on a SDS-PAGE (10% [w/v]). Proteins were transferred to a nitro-cellulose membrane for immunodetection with specific antibody raised against the C-terminal region of the recombinant plastidal Protox of spinach. The position of molecular mass markers (in kD) is given at left.

of the thylakoid membrane, and a small portion of plastidal Protox is located on the stromal side of the inner envelope membrane of chloroplasts.

DISCUSSION

In this study we have isolated the plastidal Protox cDNA, *SO-POX1*, from spinach. The plastidal Protox of spinach has a highly conserved domain in its N terminus that contains the consensus sequence GxGxxG (Gly-89 to Gly-94), which is part of the $\beta\alpha\beta$-ADP binding fold found in many flavoproteins (Wierenga et al., 1986; Dailey and Dailey, 1998). Because the Gly residues in the $\beta\alpha\beta$-ADP binding fold are believed to interact with the phosphoryl groups of the adenosine moiety of FAD, the plastidal Protox of spinach would presumably require FAD as a cofactor. We have recently reported that Protox was purified from spinach chloroplasts 1,600-fold (Watanabe et al., 2000). The activity of the purified Protox was stimulated about 1.4-fold by addition of 1 μM FAD, while NAD addition at the micromolar levels (1–10 μM) did not affect Protox activity (Watanabe et al., 2000). Such activation of Protox by FAD corresponds with the data of mammalian Protox purified from murine (Dailey and Karr, 1987). These data suggest that the plastidal Protox of spinach requires FAD as a cofactor and that FAD would presumably also bind Gly residues in the $\beta\alpha\beta$-ADP binding fold. However, we could not confirm the existence of FAD in purified Protox because the amount of purified Protox was too small to ascertain the presence of FAD.

Though our immunological studies indicate that plastidal Protox is a membrane-bound protein, no transmembrane domain has been confirmed in any known Protoxes by sequence analysis using the TMAP software (EMBL Computational Services, Cambridge, UK) (Camadro et al., 1999). Therefore, we attempted to analyze the putative transmembrane motif of the plastidal Protox of spinach using another algorithm. The hydropathy profile of the protein according to Kyte and Doolittle (1982) revealed that spinach plastidal Protox is a moderately hydrophobic protein with a putative transmembrane domain in the N-terminal region (data not shown). This transmembrane region was also predicted by PSORT (Genome NET Service, Osaka University, Japan; Nakai and Kaneshita, 1992), a commercially available subcellular localization predictor. PSORT software analysis suggested that plastidal Protox has a putative transmembrane domain at Val-81 to Ala-97 that contains the GxGxxG motif. The amino acid sequence of this predicted transmembrane domain was highly conserved among the other plant Protoxes, suggesting that they may be bound to the membrane at this transmembrane domain.

It has been reported that the typical consensus sequence of the cleavage site, (Val/Ile) $-3 - X - 2 -$ (Ala/Cys) $- 1 -$ Ala $+ 1$, is found in the majority of

Figure 7. Immunogold labeling of plastidal Protox in a spinach mesophyll cell from sponge tissue of 4-week-old seedlings. Sections were immunostained with C-terminal region specific antibody (A–C) or with preimmune serum (D). M, Mitochondrion; C, chloroplast; T, thylakoid; S, stroma; IE, inner envelope; OE, outer envelope; CW, cell wall. Bar = 0.5 μm.
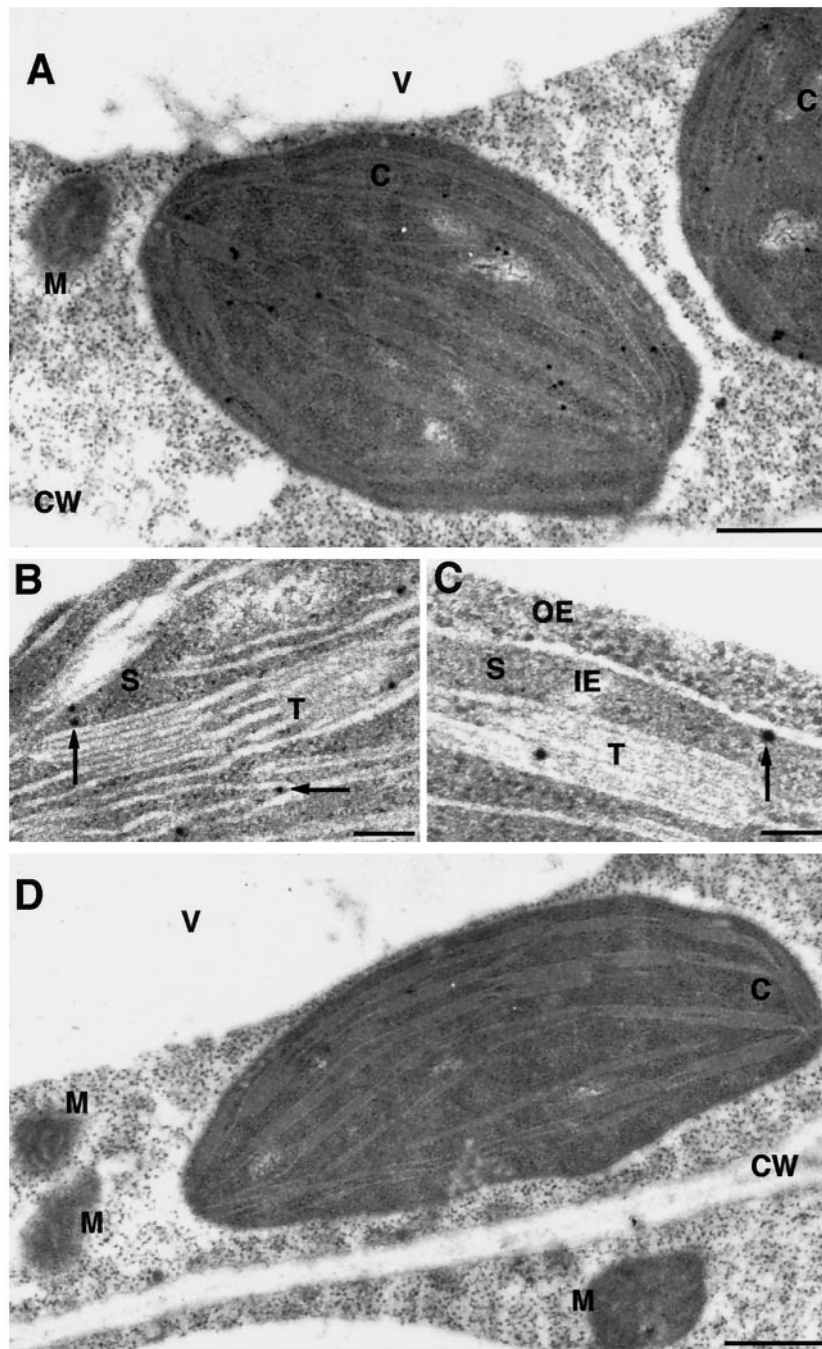
transit peptides (Gavel and von Heijne, 1990). However, this consensus sequence is not found in the deduced amino acid sequences of any known plastidal Protox precursors from higher plants, including spinach. Though the transit peptide sequences of plastidal Protoxes of various plants show no homology, three amino acids at the cleavage site are conserved in all known plastidal Protoxes (Arg-Cys-Ser; Fig. 1). Furthermore, the cleavage prediction generated by the ChloroP method (Emanuelsson et al., 1999) also revealed that the predicted cleavage site of

plastidal Protoxes is within this conserved sequence, indicating that all other plastidal Protox precursors would also be cleaved between Cys and Ser.

The results of immunogold electron microscopy showed that plastidal Protox was localized on the stromal side of the thylakoid and inner envelope. Chlorophyll and heme are synthesized from the intact carbon skeleton of Glu via the C5 pathway (Reinbothe and Reinbothe, 1996) with the initial steps of 5-aminolevulinic acid formation and the steps leading to Protogen IX likely to occur in the stroma (Smith and

Rebeiz, 1979; Kruse et al., 1995; Mock et al., 1995). Plastidal Protox is the first enzyme in the chlorophyll synthetic pathway catalyzed by membrane-bound enzymes, and all subsequent steps are thought to be catalyzed by membrane-bound enzymes (von Wettstein et al., 1995). It is reasonable to assume that plastidal Protox is located on the stromal side of both membranes because this location is very convenient to receive the substrate of Protox from coproporphyrinogen oxidase located in stroma. It is not clear whether subsequent pathway membrane-bound enzymes such as Mg-chelatase (Walker and Weinstein, 1995) or ferrochelatase (Roper and Smith, 1997; Chow et al., 1998) are located on the stromal side of the membrane. However, it seems likely that these enzymes would also be located on the stromal side of the membrane because of the rapid enzymatic cascade reaction yielding chlorophyll or heme.

Since Protox is the last common enzyme in the biosynthesis pathway of chlorophyll and heme, Protox must play the important role of supplying and distributing Proto IX to the next two enzymes, Mg-chelatase and ferrochelatase. Mg-chelatase catalyzes the insert of Mg^{2+} into Proto IX to yield Mg-Proto IX, and the activity of this enzyme is detected in envelope membranes (Walker and Weinstein, 1995). Protochlorophyllide reductase, which catalyzes the reduction of protochlorophyllide to chlorophyllide *a*, is also located in an envelope membrane (Joyard et al., 1990). Thus, the envelope membrane is the major site of chlorophyll synthesis even though the final destination of chlorophyll is the thylakoid. In contrast, ferrochelatase, which catalyzes the insertion of ferrous ions into Proto IX, is associated with the thylakoid membrane (Matringe et al., 1994), suggesting that heme synthesis mainly takes place on the thylakoid membrane. Using immunoblot and immunogold electron microscopic analyses, we have shown in this work that plastidal Protox is located on the envelope and thylakoid membranes of chloroplasts. Such dual localization of plastidal Protox implies that the Protox located on the envelope membrane supplies Proto IX to the chlorophyll biosynthetic pathway, while the Protox on the thylakoid membrane supplies Proto IX to the heme biosynthetic pathway. The distribution of tetrapyrrole to chlorophyll and heme biosynthetic pathways would be controlled at the insertion step of magnesium and ferrous ions.

The information presented in this paper about the chloroplast localization of plastidal Protox raises the question of how the product of this Protox gene is targeted to two different compartments in spinach chloroplasts, the inner envelopes and the thylakoids. Usually, most precursor proteins are larger than their corresponding plastid-localized forms. The N-terminal transit peptide that is cleaved off the precursor protein upon entry into chloroplasts usually contains stromal-targeting information that is necessary and sufficient for the transport of precur-

sors across the two envelope membranes (Keegstra et al., 1989; de Boer and Weisbeek, 1991; Keegstra and Cline, 1999). The stromal-targeting domains from various precursor proteins may vary in length from 30 to 100 amino acids (von Heijne et al., 1989; Keegstra and Cline, 1999). Most stromal-targeting domains are rich in Ser and Thr but deficient in acidic amino acids (von Heijne et al., 1989). The N-terminal domain (Met-1 to Cys-48) of the plastidal Protox precursor from spinach is rich in Ser (13 of the 48 amino acid residues are Ser) and deficient in acidic amino acids, corresponding with the above features of the stromal-targeting domain. This putative transit peptide domain of plastidal Protox was sufficient for the transport of the Protox to plastids because the GFP fused with the predicted transit peptide (Met-1 to Cys-48) was transported to the chloroplast (Fig. 5). The additional targeting information for insertion into membranes is generally contained within the mature region of the protein (Keegstra and Cline, 1999). This domain is composed of hydrophobic amino acids, and the targeting information is generally located in membrane-spanning domains (Cline and Henry, 1996; Keegstra and Cline, 1999). The additional targeting domain of spinach plastidal Protox for insertion into the envelope and thylakoid membrane is still not known, but it seems likely that the predicted transmembrane domain (Val-81 to Ala-97) confirmed by the PSORT program is important for plastidal Protox insertion.

MATERIALS AND METHODS

Chemicals and Plant Materials

Spinach plants (*Spinacia oleracea* L. cv Tonic; Watanabe-Saishujyo Ltd., Japan) were grown in a greenhouse at 25°C for 8 weeks or in a growth chamber on a 16-h/8-h cycle (day/night) at 25°C for 4 weeks at a light intensity of 140 μmol m^{-2} s^{-1}. The reagents used were of special or analytical grade from Wako Pure Chemicals (Osaka) and Nakarai Tesque Company (Kyoto). Herbicide S-23142 was a gift from Sumitomo Chemical Company (Takarazuka, Japan).

cDNA Cloning and Sequence Analysis

Total RNA was isolated using RNeasy Plant Kits (Qiagen, Hilden, Germany) from 4-week-old spinach leaves. First-strand cDNA was synthesized from total RNA using a Ready-To-Go T-primed First-Strand Kit (Amersham Pharmacia Biotech, Piscataway, NJ). For PCR isolation of the plastidal Protox gene, one set of oligonucleotide primers was synthesized on the basis of the nucleotide sequences of Protox cDNAs conserved in Arabidopsis (Narita et al., 1996) and tobacco (*Nicotiana tabacum* cv Samsun NN; Lermontova et al., 1997). The sequences of the primer set were 5′-CTTGGTGGCGAAGTCTTTGAA-3′ (SCP-1F) and 5′-CCAATGCTACACCTGACACATA-3′ (SCP-2R). *Taq*-DNA polymerase and a buffer (Expand High Fidelity

PCR system) were purchased from Boehringer Mannheim (Mannheim, Germany) and used for all PCR experiments (94°C for 5 min, 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C). PCR reactions were terminated with 10 min of incubation at 72°C and stored at 4°C. PCR fragments (0.9 kb) were cloned using a TA cloning kit (Invitrogen, Groningen, Netherlands), and five clones from each PCR reaction were sequenced with a DNA sequencer (model 377; Perkin-Elmer Applied Biosystems, Foster City, CA). PCR and cloning procedures were independently repeated twice to confirm DNA sequences.

The 5′ end of Protox cDNA was amplified by RACE using a 5′/3′ RACE kit (Boehringer Mannheim). First-strand cDNA synthesized from mRNA was dA-tailed with terminal deoxytransferase, and second-strand cDNA was synthesized using a poly(T) cassette primer (5′-ACTC-GAATTCACGCGGCCGCAT15–3′). A first 5′-RACE PCR was performed using specific primer I (5′-CCTGCCAAAAG-CAGCTTTCAT-3′) and cassette primer I (5′-ACTCGAATTC-ACGCGGCCGCA-3′) with the recommended PCR condition. After the first reaction the PCR product was diluted 2,000-fold and subjected to secondary PCR amplification. The second amplification was performed using specific primer II (5′-ATCTCCCAGCACCAATTCCTCT-3′) and cassette primer I. For 3′-RACE, cDNA was synthesized from mRNA with a poly(T) cassette primer (5′-TGG-AAGAATTCGCGGCGGCAGT16–3′). 3′-RACE PCR was performed using specific primer III (5′-GTGAGAGTGGC-CACAAGCAA-3′) and cassette primer II (5′-TGGAA-GAATTCGCGGCGGCAG-3′). The amplified fragments for 3′ RACE (0.65 kb) and for 5′ RACE (0.8 kb) were cloned with a TA cloning kit (Invitrogen), and five independent clones from each PCR reaction were sequenced as described above. PCR and cloning procedures were independently repeated twice, and DNA inserts were sequenced on both strands to ensure that no mutation had been introduced during PCR amplification.

Southern-Blot Analysis

Genomic DNAs were isolated from young spinach leaves by Nucleon plant-DNA extraction kits (Amersham Pharmacia Biotech) according to the standard protocol. The DNAs (10 μg) were digested with *Bgl*II, *Dra*I, or *Eco*RI and then electrophoresed on a 0.7% (w/v) agarose gel. Fractionated DNA was transferred onto a Hybond N$^+$ membrane (Amersham Pharmacia Biotech). Plastidal Protox cDNA (1.8 kb) was labeled with an AlkPhos Direct labeling kit (Amersham Pharmacia Biotech) according the manufacturer's instructions. Hybridization was performed at 65°C, and the signals were detected by a chemiluminescent detection reagent system (CDP-Star, Amersham Pharmacia Biotech).

Antibody Production and Purification

The plastidal Protox C-terminal region derived from Ser-303 to Lys-562 (Fig. 1) was cloned in-frame in the pET-28(a)$^+$ vector (Novagen, Madison, WI). The resulting

His-tag fusion protein was overproduced in the BL21(DE3) strain of *Escherichia coli* (Invitrogen) and purified using a HisTrap kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). The protein was further purified on a HiTrap SP column (5 mL; Amersham Pharmacia Biotech), and the purified recombinant protein (10 mg) was used to immunize rabbits. Anti-serum was further purified by affinity chromatography with a HiTrap NHS-activated Sepharose column (Amersham Pharmacia Biotech).

Immunoblot Analysis

For immunoblot analysis, intact chloroplasts and mitochondria were isolated from 4-week-old spinach leaves by centrifugation on a Percoll linear gradient according to the method of Gualberto et al. (1995). Intactness of chloroplasts and mitochondria was judged by the activity of ferricyanide reduction (Lilley et al., 1975) and by the latency of cytochrome c oxidase activity (Krömer and Heldt, 1991). For purification of the stroma, envelope, and thylakoid fractions from intact chloroplasts, 15 mL of two sets of dicontinuous Suc gradients (0.6 and 0.98 м) dissolved in hypotonic buffer (10 mм Tricine [*N*-(2-hydroxy-1,1-Bis[hydroxymethyl]ethyl)glycine]-KOH, pH 7.8, 4 mм MgCl$_2$, and 1 mм phenylmethylsulfonyl fluoride [PMSF]) were prepared (Joyard et al., 1982). A 5-mL suspension of intact chloroplasts osmotically lysed in hypotonic buffer was layered onto the gradients, and the tubes were ultracentrifuged at 90,000g for 90 min at 4°C. After centrifugation, three fractions were clearly separated: a dark green pellet at the bottom of the tube (thylakoid fraction); a yellow band at the interface of the two Suc layers (envelope fraction); and a slightly yellow supernatant (stroma fraction). These samples were separated by SDS-polyacrylamide gels (12.5%) as described by Laemmli (1970). Separated proteins were electrophoretically transferred to a nitrocellulose membrane (Millipore, Bedford, MA) with a semidry blotter (Bio-Rad Laboratories, Hercules, CA). Non-specific binding was blocked with 3% (w/v) bovine serum albumin (BSA) in PBS buffer for 1 h at room temperature. Immunoreactive polypeptides were detected using an alkaline phosphatase-conjugated goat antibody raised against rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized by reaction with nitroblue tetrazolium chloride and bromochloroindonyl phosphate.

Purification and Determination of the N-Terminal Sequence

Spinach leaves (2.4 kg) were harvested and kept in a cold room for 2 d in the dark. The leaves were subsequently irradiated with light (120 μmol m^{-2} s^{-1}) for 2 h before extraction of chloroplasts. Spinach chloroplasts were isolated by the method of Wang et al. (1993) with slight modifications. Spinach leaves were cut into small pieces, directly immersed in an ice-cold extraction medium (330 mм Suc, 1 mм EDTA, 1 mм MgCl$_2$, 0.1% [w/v] BSA, 5 mм Cys hydrochloride monohydrate, 20 mм TES [*N*-Tris(hydroxy-

methyl)-2-aminoethanesulfonic acid], and 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] adjusted to pH 7.9 with KOH) and homogenized for 5 s with a Polytron (PT 10/35; Kinematica, Westbury, NY). After filtration through four layers of gauze, the chloroplasts were pelleted by centrifugation at 2,500g for 3 min. The pellet was gently resuspended and centrifuged at 150g for 2 min, and then the supernatant was centrifuged at 2,000g for 1 min. Intact chloroplasts were osmotically broken by resuspension in a 1:13 dilution of a chloroplast extraction buffer plus 0.8 mM PMSF and centrifuged at 10,000g for 30 min. The resulting pellets contained chloroplast membranes (thylakoids and envelope). Protox activity was solubilized by diluting the membrane fraction to a protein concentration of 5 mg/mL in a 1:13 dilution of a chloroplast extraction buffer plus 0.8 mM PMSF and 0.1% (w/v) Triton X-100. The membrane suspension was gently stirred for 1 h, and then the insoluble material was removed by ultracentrifugation at 105,000g for 1 h. Protox activity was recovered in the soluble fraction, and the resultant soluble extract was used as crude extract.

Additional purification of plastidal Protox was performed by the immunoprecipitation method. The crude extract was incubated with 15 μg of specific antibody against Protox at room temperature for 3 h and further incubated with 50 μL of Protein A-beads (Pierce Chemical, Rockford, IL) at 4°C overnight. The complex of Protein-A beads and antibody protein was collected by centrifugation (2,000g, 10 s). The pellet was washed twice with a cold Tris (tris[hydroxymethyl]aminomethane)-buffered saline plus Triton X-100 buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% [w/v] Triton X-100) and further washed with cold distilled water. The bead complex was suspended in 50 μL of the sample buffer for SDS-PAGE and analyzed on a 10% (w/v) gel according to the method of Laemmli (1970). The proteins in the gel were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore) using Transblot SD (Bio-Rad Laboratories) at 10 V for 2 h. After staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, the visualized band (60 kD) was excised and the N-terminal sequence was determined with a pulse liquid-phase protein sequencer (Perkin-Elmer Applied Biosystems, model 492A). Protein contents were determined by a Protein assay kit (Bio-Rad Laboratories) using BSA as a standard.

Functional Complementation of *hemG*-Deficient Mutant of *E. coli*

The full-length (Met-1 to Lys-563) and mature forms (Ser-49 to Lys-563) of plastidal Protox cDNA clones were amplified by PCR using two sets of specific primers, (5′-TATGAGCGCTATGGCGTTATCGAG-3′ and 5′-CTATTT-ATCCGAGTACTGTGACAGAA-3′) and (5′-TTCTACAA-TCTCAACCTCTAATTCC-3′ and 5′-CTATTTATCCGAGT-ACTGTGACAGAA -3′), respectively. Amplified fragments were cloned into a pCR 2.1 plasmid vector (Invitrogen) in-frame with the *LacZ* gene. The resulting plasmids, pP-pProtox (for the precursor form) and pM-pProtox (for

the mature form), were introduced into the *E. coli* BT3 (ΔhemG::Km^r), which is obtained by replacement from the wild-type *hemG* gene to the *hem*G::Km^r allele by homologous recombination (Narita et al., 1999). For a positive control, the *hemG* gene of *E. coli* (Nishimura et al., 1995a) was cloned into a pCR 2.1 vector and transformed into BT3 (ΔhemG::Km^r) cells in the same manner. Transformants were plated on Luria-Bertani (LB) plates containing 1% (w/v) bactotrypton, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 50 μg/mL ampicillin, and 25 μg/mL kanamycin at 37°C. Complemented cells produced normal-size colonies, and the other cells produced dwarf colonies on the plate for 2-d culture. Each plasmid isolated from these complemented cells was re-introduced into BT3 (ΔhemG::Km^r) cells and confirmed to complement the mutation.

Transport Experiment Using a Green Fluorescent Protein Fusion

For the construction of GFP expression vector, GFP coding sequence was PCR-amplified from pGFP2 vector (P. Spielhofer and N.-H. Chua, unpublished data) using one set of specific primers, (5′-AGTCACTAGTATGGGTA-AGGGAGAAGAACTTTTC-3′, 5′-CAGTGAGCTCGATTT-GTATAGTTCATCCATGCCA-3′) and the amplified fragments were cloned into a pCR 2.1 plasmid vector (Invitrogen). The targeting sequences corresponding to the upstream region of the processing point (Met-1 to Cys-48) and the flanking 25 amino acid residues (Met-1 to Asp-73) were PCR-amplified from the Protox cDNA using pairs of primers as follows: Met-1 to Cys-48, 5′-ACTGCCATGGATG-AGCGCTATGGCGTTATCG-3′ and 5′-CAGTACTAGTG-CAGCGGATAGAGCTTCCTC-3′; Met-1 to Asp-73), 5′-AC TGCCATGGATGAGCGCTATGGCGTTATCG-3′ and 5′-CAGTACTAGTGTCAACTCCGTTTGTGCCTA-3′. Amplified fragments were digested with *Nco*I and *Spe*I and cloned in-frame into the *Nco*I and *Spe*I sites of the pCR 2.1 vector contained with *GFP2* gene. The plasmid was further digested with *Nco*I and *Sac*I and cloned into the *Nco*I and *Sac*I sites of the expression vector pBI221 (Novagen). Gold particles (2.5 μg of 1-μm gold particles) were coated with 2.5 μg of the constructed plasmid DNAs by CaCl$_2$/spermidine precipitation as previously described (Cao et al., 1992). Spinach leaves were bombarded with the gold particles using a particle gun (Bio-Rad Laboratories PDS-100/He). The distance between the loaded DNA and the target leaves was 8 cm, and the pressure used was 1,100 psi. After overnight incubation at 25°C, transient expression was observed using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with Micro Mover-W (Photometrics, Tucson, AZ) fitted with a triple-band filter (no. 81 series PINKEL no. 1 Filter SET, Chroma Technology, Brattleboro, VT). Autofluorescence was observed in chloroplasts at excitation wavelength of 540 nm and emission wavelength of 600 nm, and the fluorescence of GFP was observed at excitation wavelength of 495 nm and emission wavelength of 530 nm. Two images were acquired separately in the IP Lab-PVCAM system through a cooled CCD camera (Photometrics) and pseudocolored

based on the original emission fluorescence. The composite images were printed with Pictography (Fuji, Tokyo).

Immunogold Electron Microscopy

For immunogold electron microscopy of plastidal Protox distribution, spinach leaves of 1-month-old seedlings were fixed with 0.6% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in a 0.05 M sodium cacodylate buffer (pH 7.4) for 5 min at 4°C under a vacuum and stored at 4°C for 1 h. The tissues were rinsed with a 0.05-M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. The fixed materials were then dehydrated in an ethanol series at 4°C, embedded in LR White resin (The London Resin Co., London), and polymerized by UV irradiation at room temperature (Suzaki and Kataoka, 1992; Tomoyasu et al., 1993). Ultrathin sections were cut with a diamond knife and mounted on uncoated nickel grids.

Prior to incubation in the primary antibody, the sections were blocked with 5% (w/v) BSA in PBS for 30 min. Specific antibody against plastidal Protox was diluted in PBS supplemented with 1% (w/v) BSA. Incubation with the primary antibody was performed overnight at 4°C followed by washing with 0.05% (w/v) Tween 20 in PBS (PBST, pH 7.4). The secondary antibody, goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles (Biocell Research Laboratories, Cardiff, UK), was diluted in PBST. Sections were incubated in the secondary antibody for 30 min at 37°C. The sections were then washed in PBST and in distilled water, followed by staining with uranyl acetate for 15 min. The sections were observed under a transmission electron microscope (H-7100; Hitachi, Tokyo) at an accelerating voltage of 75 kV.

LITERATURE CITED

Arnould S, Berthon J-E, Hubert C, Cibert C, Mornet R, Camadro JM (1997) Kinetics of protoporphyrinogen oxidase inhibition by diphenyleneiodonium derivatives. Biochemistry **36:** 10178–10184

Bach AW, Lan NC, Johnson DL, Abell CW, Bembenek ME, Kwan SW, Seeburg PH, Shih JC (1988) cDNA cloning of human liver monoamine oxidase A and B: molecular basis of differences in enzymatic properties. Proc Natl Acad Sci USA **85:** 4934–4938

Beale SI, Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organism. *In* HA Dailey, ed, Biosynthesis of Heme and Chlorophylls. McGraw-Hill, New York, pp 287–391

Birchfield NB, Latli B, Casida JE (1998) Human protoporphyrinogen oxidase: relation between the herbicide binding site and flavin cofactor. Biochemistry **37:** 6905–6910

Camadro JM, Arnould S, Le Guen L, Santos R, Matringe M, Mornet R (1999) Characteristics of protoporphyrinogen oxidase. *In* P Böger, K Wakabayashi, eds, Peroxidizing Herbicides. Springer, Berlin, pp 245–277

Camadro JM, Labbe P (1996) Cloning and characterization of the *HEM14* gene coding for protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. J Biol Chem **271:** 9120–9128

Camadro JM, Matringe M, Scalla R, Labbe P (1991) Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. Biochem J **277:** 17–21

Cao J, Xiaolan D, David M, Ray W (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep **11:** 586–591

Che FS, Takemura Y, Suzuki N, Ichinose K, Wang JM, Yoshida S (1993) Localization of target-site of protoporphyrinogen oxidase-inhibiting herbicides, S-23142, in *Spinacia oleracea* L. Z Naturforsch **48c:** 350–355

Chow KS, Singh DP, Walker AR, Smith AG (1998) Two different genes encode ferrochelatase in Arabidopsis: mapping, expression and subcellular targeting of the precursor proteins. Plant J **15:** 531–541

Cline K, Henry R (1996) Import and routing of nucleus-encoded chloroplast proteins. Annu Rev Cell Dev Biol **12:** 1–26

Dailey HA (1990) Conversion of coproisoporphyrinogen to protoheme in higher eukaryotes and bacteria. *In* HA Dailey, ed, Biosynthesis of Heme and Chlorophylls. McGraw-Hill Publishing Co., New York, pp 123–161

Dailey HA, Karr SW (1987) Purification and characterization of murine protoporphyrinogen oxidase. Biochemistry **26:** 2697–2701

Dailey TA, Dailey HA (1998) Identification of an FAD superfamily containing protoporphyrinogen oxidases, monoamine oxidases and phytoene desaturase: expression and characterization of phytoene desaturase of *Myxococcus xanthus*. J Biol Chem **273:** 13658–13662

de Boer AD, Weisbeek PJ (1991) Chloroplast protein topogenesis: import, sorting and assembly. Biochim Biophys Acta **1071:** 221–253

Duke SO, Becerril JM, Sherman TD, Matsumoto H (1991) Photosensitizing porphyrins as herbicides. Weed Sci **39:** 465–473

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci **8:** 978–984

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

Grimm B (1998) Novel insight in the control of tetrapyrrole metabolism of higher plants. Curr Opin Plant Biol **1:** 245–250

Gualberto JM, Handa H, Grienenberger JM (1995) Isolation and fractionation of plant mitochondria and chloroplasts: specific example. Method Cell Biol **50:** 161–175

Hansson M, Hederstedt L (1994) *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coprotoporphyrinogen III and protoporphyrinogen IX. J Bacteriol **176:** 5962–5970

Ichinose K, Che FS, Kimura Y, Matsunobu A, Sato F, Yoshida S (1995) Selection and characterization of protoporphyrinogen oxidase inhibiting herbicide (S23142) resistant photomixotrophic cultured cells of *Nicotiana tabacum.* J Plant Physiol **146:** 693–698

Jacobs JM, Jacobs NJ (1987) Oxidation of protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis. Biochem J **244:** 219–224

Jacobs JM, Jacobs NJ (1993) Porphyrin accumulation and export by isolated barley (*Hordeum vulgare*) plastids. Plant Physiol **101:** 1181–1187

Jacobs JM, Jacobs NJ, Sherman TD, Duke SO (1991) Effect of diphenyl ether herbicides on oxidation of protoporphyrinogen to protoporphyrin in organellar and plasma membrane enriched fractions of barley. Plant Physiol **97:** 197–203

Johnston DJ, Droz E, Rochaix J-D, Malno P (1998) Cloning and characterization of potato cDNAs involved in tetrapyrrole biosynthesis (accession nos. ferrochelatase AJ005802, chloroplastic protoporphyrinogen IX oxidase AJ225107, mitochondrial protoporphyrinogen IX oxidase AJ225108) (PGR 98–156). Plant Physiol **118:** 330

Joyard J, Block M, Pineau B, Albrieux C, Douce R (1990) Envelope membranes from mature spinach chloroplasts contain a NADPH: protochlorophyllide reductase on the cytosolic side of the outer membrane. J Biol Chem **265:** 21820–21827

Joyard J, Grossman A, Bartlett SG, Douce R, Chua NH (1982) Characterization of envelope membrane polypeptides from spinach chloroplasts. J Biol Chem **257:** 1095–1101

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell **11:** 557–570

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol **40:** 471–501

Krömer S, Heldt HW (1991) Respiration of pea leaf mitochondria and redox transfer between the mitochondria and extramitochondrial compartment. Biochim Biophys Acta **1057:** 42–50

Kruse E, Mock HP, Grimm B (1995) Coprotoporphyrinogen III oxidase from barley and tobacco: sequence analysis and initial expression studies. Planta **196:** 796–803

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:** 105–132

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lee HJ, Duke MV, Duke SO (1993) Cellular localization of protoporphyrinogen-oxidizing activities of etiolated barley (*Hordeum vulgare* L.) leaves. Plant Physiol **102:** 881–889

Lee HJ, Duke SO (1994) Protoporphyrinogen IX-oxidizing activities involved in the mode of action of peroxidizing herbicides. J Agric Food Chem **42:** 2610–2618

Lermontova I, Kruse E, Mock HP, Grimm B (1997) Cloning and characterization of a plastidal and a mitochondrial isoforms of tobacco protoporphyrinogen IX oxidase. Proc Natl Acad Sci USA **94:** 8895–8900

Lilley RM, Fitzgerald MP, Rienits KG, Walker DA (1975) Criteria of intactness and photosynthetic activity of spinach chloroplast preparations. New Phytol **75:** 1–10

Matringe M, Camadro JM, Block MA, Joyard J, Scalla R, Labbe P, Douce R (1992b) Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether-type herbicides. J Biol Chem **267:** 4646–4651

Matringe M, Camadro JM, Joyard J, Douce R (1994) Localization of ferrochelatase activity within mature pea chloroplasts. J Biol Chem **269:** 15010–15015

Matringe M, Camadro JM, Labbe P, Scalla R (1989) Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. Biochem J **260:** 231–235

Matringe M, Mornet R, Scalla R (1992a) Characterization of [^{3}H]acifluorfen binding to purified pea etioplasts, and evidence that protoporphyrinogen oxidase specifically binds acifluorfen. Eur J Biochem **209:** 861–868

Mock HP, Trainotti L, Kruse E, Grimm B (1995) Isolation, sequencing and expression of cDNA sequences encoding uroporphyrinogen decarboxylase from tobacco and barley. Plant Mol Biol **28:** 245–256

Molina A, Volrath A, Guyer D, Maleck K, Ryals J, Ward E (1999) Inhibition of protoporphyrinogen oxidase expression in *Arabidopsis* causes a lesion-mimic phenotype that induces systemic acquired resistance. Plant J **17:** 667–678

Nakai K, Kaneshita M (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14:** 897–911

Narita S, Taketani S, Inokuchi H (1999) Oxidation of protoporphyrinogen IX in *Escherichia coli* is mediated by the aerobic coproporphyrinogen oxidase. Mol Gen Genet **261:** 1012–1020

Narita S, Tanaka R, Ito T, Okada K, Taketani S, Inokuchi H (1996) Molecular cloning and characterization of cDNA that encodes protoporphyrinogen oxidase of *Arabidopsis thaliana*. Gene **182:** 169–175

Nishimura K, Nakayashiki T, Inokuchi H (1995a) Cloning and identification of the *hemG* gene encoding protoporphyrinogen oxidase (PPO) of *Escherichia coli* K-12. DNA Res **2:** 1–8

Nishimura K, Taketani S, Inokuchi H (1995b) Cloning of a human cDNA for protoporphyrinogen oxidase by complementation *in vivo* of a *hemG* mutant of *Escherichia coli*. J Biol Chem **270:** 8076–8080

Reinbothe S, Reinbothe C (1996) The regulation of enzymes involved in chlorophyll biosynthesis. Eur J Biochem **237:** 323–343

Roper JM, Smith AG (1997) Molecular localization of ferrochelatase in higher plant chloroplasts. Eur J Biochem **246:** 32–37

Sasarman A, Letowski J, Czaika G, Ramirez V, Nead MA, Jacobs JM, Morais R (1993) Nucleotide sequence of the *hemG* gene involved in the protoporphyrinogen activities of *Escherichia coli* K12. Can J Microbiol **39:** 1155–1161

Sato R, Nagao E, Ohshio H, Kamoshita K (1987) Diphenyl ether-like physiological and biochemical action of S23142, a novel *N*-phenylimide herbicides. Pestic Biochem Physiol **28:** 194–200

Scalla R, Matringe M, Camadro JM, Labbe P (1990) Recent advance in the mode of action of diphenyl ether and related herbicides. Z Naturforsch **45c:** 503–511

Smith AG, Marsh O, Elder GH (1993) Investigation of the subcellular location of the tetrapyrrole-biosynthesis enzyme coproporphyrinogen oxidase in higher plants. Biochem J **292:** 503–508

Smith BB, Rebeiz CA (1979) Chloroplast biogenesis: intrachloroplastic localization of the biosynthesis and accumulation of protoporphyrin IX, magnesium-protoporphyrin monoester, and longer wavelength metalloporphyrin during greening. Plant Physiol **63:** 227–231

Suzaki E, Kataoka K (1992) Lectin cytochemistry in the gastrointestinal tract with special reference to glycosylation in the Golgi apparatus of Brunner's gland cells. J Histochem **40:** 379–385

Tomoyasu T, Yamanaka K, Murata K, Suzaki T, Bouloc P, Kato A, Niki H, Hiraga S, Ogura T (1993) Topology and subcellular localization of FtsH protein in *Escherichia coli*. J Bacteriol **175:** 1352–1357

Varsano R, Matringe M, Magnin N, Mornet R, Scalla R (1990) Competitive interaction of three peroxidizing herbicides with the binding of [³H]acifluorfen to corn etioplast membrane. FEBS Lett **272:** 106–108

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

von Wettstein D, Gough S, Kannangara CG (1995) Chlorophyll biosynthesis. Plant Cell **7:** 1039–1057

Walker CJ, Weinstein JD (1995) Re-examination of the localization of Mg-chelatase within the chloroplasts. Physiol Plant **84:** 419–424

Wang JM, Che FS, Asami T, Murohushi N, Yoshida S (1993) New assay method for protoporphyrinogen oxidase inhibitor using chloroplasts isolated from *Spinacia oleracea* L. Biosci Biotech Biochem **57:** 2205–2206

Watanabe N, Che FS, Iwano M, Takayama S, Nakano T, Yoshida S, Isogai A (1998) Molecular characterization of photomixotrophic tobacco cells resistant to protoporphyrinogen oxidase-inhibiting herbicides. Plant Physiol **118:** 751–758

Watanabe N, Che FS, Terashima K, Takayama S, Yoshida S, Isogai A (2000) Purification and properties of protoporphyrinogen oxidase from spinach chloroplasts. Plant Cell Physiol **41:** (in press)

Wierenga RK, Terpstra P, How WGI (1986) Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. J Mol Biol **187:** 101–107

Witkowski DA, Halling BP (1989) Inhibition of plant protoporphyrinogen oxidase by the herbicides acifluorfen methyl. Plant Physiol **90:** 1239–1242