Dual Targeting of Spinach Protoporphyrinogen Oxidase II to Mitochondria and Chloroplasts by Alternative Use of Two In-frame Initiation Codons*  

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Protox is the final enzyme in the common pathway of chlorophyll and heme biosynthesis. Two Protox isoenzymes have been described in tobacco, a plastidic and a mitochondrial form. We isolated and sequenced spinach Protox cDNA, which encodes a homolog of tobacco mitochondrial Protox (Protox II). Alignment of the deduced amino acid sequence between Protox II and other tobacco mitochondrial Protox homologs revealed a 26-amino acid N-terminal extension unique to the spinach enzyme. Immunoblot analysis of spinach leaf extract detected two proteins with apparent molecular masses of 57 and 55 kDa in chloroplasts and mitochondria, respectively. In vitro translation experiments indicated that two translation products (59 and 55 kDa) are produced from Protox II mRNA, using two in-frame initiation codons. Transport experiments using green fluorescent protein-fused Protox II suggested that the larger and smaller translation products (Protox III and IIS) target exclusively to chloroplasts and mitochondria, respectively.

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1 The abbreviations used are: Protox, protoporphyrinogen oxidase; bp, base pairs; kb, kilobase pair(s); mlb, kilobase(s); BSA, bovine serum albumin; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween 20; PCR, polymerase chain reaction; PPX-II, tobacco mitochondrial protoporphyrinogen oxidase; Protox IX, protoporphyrinogen oxidase; Protox XII, the larger form of Protox II; Protox III, the smaller form of Protox II; RACE, rapid amplification of cDNA ends; S23142, N-[4-chloro-2-fluoro-5-propargyloxy]-phenyl-3,4,5,6-tetrahydrophthalimide.
associated with this structure. In plant cells, Protox activity and tobacco PPX-II protein itself have also been observed in mitochondria (13). However, there have been no investigations into the precise suborganellar location of Protox in plant mitochondria. Furthermore, little is known about the transport mechanism of mitochondrial Protox. In general, proteins transported into the mitochondria have an N-terminal targeting peptide, which is processed after transport is complete (16). However, tobacco PPX-II and other known homologs of PPX-II do not possess the typical mitochondrial targeting sequence at their N termini (Ref. 17; accession nos. AB025102 and AJ225107). Actually, in vitro transport experiments have shown that tobacco PPX-II is transported to mitochondria without any size reduction (13). The mechanism by which this occurs is not yet understood.

Protox is the target enzyme of phthalimide-type herbicides such as N-[4-chloro-2-fluoro-5-propagoyloxy]-phenyl]-3,4,5,6-tetrahydrophthalimide (S23142) and diphenylether-type herbicides such as 5-[2-chloro-4-(trifluoromethyl) phenoxy]-2-nitrobenzoic acid (18). In plant cells, the inhibition of Protox by the herbicides ultimately results in massive accumulation of Protox, which then leaks out of the plastid (19). Cytosolic proteggon is rapidly oxidized to Proto IX by nonspecific, herbicide-resistant peroxidases that are bound to the plasma membrane. Highly reactive singlet oxygen generated by light activation of Proto IX provokes membrane lipid peroxidation, leading to cell death (18). We have previously obtained S23142-resistant tobacco-cultured cells (YZI-1S) by stepwise selection with the herbicide (20). Several lines of data provide strong evidence that the herbicide resistance of YZI-1S cells is due to the overproduction of mitochondrial Protox, even though the primary target of the herbicide is chloroplast Protox. In the resistant cells, excess Protox generated by inhibition of chloroplast Protox is rapidly utilized for heme synthesis in mitochondria by the abnormally high level of mitochondrial Protox, thus preventing the accumulation of Proto IX (21). This suggests that mitochondrial Protox is also important in preventing the accumulation of the photosensitizer, Proto IX, and in maintaining the flow of tetrapyrrrole precursors for heme and chlorophyll biosynthesis. Therefore, detailed knowledge about the subcellular localization and transport mechanism of plant mitochondrial Protox is important for clarification of not only the control mechanism of tetrapyrrrole biosynthesis but also the mode of action of the herbicide resistance.

In the present study, we report the molecular cloning, subcellular localization, and transport mechanism of spinach Protox II, which has high sequence identity to the tobacco mitochondrial Protox. Surprisingly, this protein was located not only in mitochondria but also in chloroplasts. Two translation products of different sizes (Protox III and Protox IIS) were produced from Protox II mRNA using two in-frame initiation codons and then were transported into chloroplasts and mitochondria, respectively. It has been thought that the known plant proteins that are dually targeted to chloroplasts and mitochondria have an ambiguous N-terminal targeting sequence, which interacts nonspecifically with import machinery (or chaperon) for both mitochondria and chloroplasts (22). The manner of the dual targeting in Protox II is clearly different from the above system. We have shown here for the first time the existence of a novel mechanism of intracellular targeting for compartmentalizing protein isoforms in chloroplasts and mitochondria.

EXPERIMENTAL PROCEDURES

Chemicals and Plant Material—Spinach plants (Spinacia oleracea L. cv. tonica, Watanabe-saishujyo Ltd.) were grown in a greenhouse at 25 °C for 8 weeks or in a growth chamber on a 12-h light/12-h dark cycle at 20 °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, Japan) and Nakarai Tesque Co. Ltd. (Kyoto, Japan). cDNA Cloning and Sequence Analysis—Total RNA was isolated from 4-week-old spinach leaves using RNeasy plant kits (Qiagen, Germany). First-strand cDNA was synthesized from total RNA using a To-Go T-primed first-strand kit (Amersham Pharmacia Biotech). For polymerase chain reaction (PCR) isolation of mitochondrial Protox cDNA, one set of oligonucleotide primers was synthesized on the basis of the nucleotide sequences of tobacco PPX-II cDNA (13, 21). The primer sequences were as follows: 5'-GAAGGAGAAGCACAACCTTACCTATGGATCAGGAGATG-3' (SMT-1R) and 5'-GATCCTCAACTTGTTCCGCA-3' (SMT-1F). Tag DNA polymerase and reaction buffer (Expand High Fidelity PCR system) were purchased from Roche Molecular Biochemicals (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 30 s at 72 °C). PCR reactions were terminated with a 10-min incubation at 72 °C and stored at 4 °C. PCR fragments (0.56 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; PE Applied Biosystems). PCR and cloning procedures were independently repeated twice to confirm DNA sequences.

The 5′ end of Protox cDNA was amplified by rapid amplification of cDNA ends (RACE) using a 5′-RACE kit (Roche Molecular Biochemicals). cDNA was synthesized from mRNA by random priming with a Cy3-labeled deoxytransferase, and second-strand cDNA was synthesized using a poly(T) cDNA polymerase. An initial 5′-RACE PCR was performed using specific primer I (5'-GATCTTCACACCTTCTTCCG-3') and primer II (5'-ACTGGAAATTCGCCGAGGACT-3'). A series of 5′-RACE PCRs was performed using specific primer I (5'-GATCTTCACACCTTCTTCCG-3') and primer II (5'-ACTGGAAATTCGCCGAGGACT-3'). The amplified fragments from 3′-RACE (1.1 kb) and 5′-RACE (0.65 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 mutations had been introduced during PCR amplification.

Southern Blot Analysis—Genomic DNA was isolated from young spinach leaves with a Nucleon plant DNA extraction kit (Amersham Pharmacia Biotech) according to standard protocol. The DNA (10 μg) was digested with BglII, DraI, or EcoRI and then electrophoresed on a 0.7% (w/v) agarose gel. Fractionated DNA was transferred onto a Hydro bond N+ membrane (Amersham Pharmacia Biotech). Mitochondrial Protox cDNA (1.6 kb) was labeled with an AlkPhos Direct labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The purified recombinant protein (10 mg) was used to immunize rabbits. Antiserum was further purified by affinity chromatography with a HiTrap N-hydroxysuccinimide-activated Sepharose column (Amersham Pharmacia Biotech) conjugated to the recombinant Protox II. Production and purification of polyclonal antibodies raised against recombinant spinach Protox I has been described previously (14).

Antibody Production and Purification—The internal region of spinach Protox II, encompassing the region from Ser-75 to Ser-415 (see Fig. 1), was cloned in-frame into the pET-28a(+) vector (Novagen, Madison, WI). The resulting His tag fusion protein was overproduced in the BL21(DE3) strain of E. coli (Invitrogen) and purified using a HisTrap kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). The purified recombinant protein (10 mg) was used to immunize rabbits. Antiserum was further purified by affinity chromatography with a HiTrap N-hydroxysuccinimide-activated Sepharose column (Amersham Pharmacia Biotech) conjugated to the recombinant Protox II. Production and purification of polyclonal antibodies raised against recombinant spinach Protox I has been described previously (14).

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 Guaitiero et al. (23). Fractionated chloroplasts and mitochondria were judged to be intact by methods described previously (14). Isolation of the envelope and thylakoid membranes from intact chloroplasts was performed as described previously (14). These samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10%, w/v). Separated proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond N-ECL, Amersham Pharmacia Biotech) with a Bio-Rad semidry blotter. Nonspecific binding was blocked with 3% bovine serum
albumin (BSA) in 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% (w/v) Tween 20 for 1 h at room temperature. Immuno-reactive polypeptides were detected using an alkaline phosphatase-conjugated goat antibody raised against rabbit IgG (H + L) (Jackson Immunoresearch Laboratories) and visualized by reaction with nitro blue tetrazolium chloride and bromochloroindolyl phosphate. Protein purification was determined with a protein assay kit (Bio-Rad) using BSA as the standard.

For quantitative analysis of the amount of Protox II and Protox I within mitochondria or chloroplasts, immunodetections were performed with an enhanced immunochemiluminescence kit (ECL-plus, Amersham Pharmacia Biotech) and chemiluminescence was detected with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The sections were then washed in PBST and in distilled water, then fixed with 0.6% glutaraldehyde and 4% paraformaldehyde in a copy of Protox II distribution, spinach leaves of 1-month-old seedlings were stained with uranyl acetate for 20 min. The sections were observed under a transmission electron microscope (H-7100, Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV.

RESULTS

Isolation and Characterization of Spinach cDNA Encoding a Tobacco Mitochondrial Protox Homolog—To isolate cDNA encoding the spinach homolog of tobacco mitochondrial Protox, one set of specific oligonucleotide primers (SMT-1F and SMT-1R) was synthesized based on the PPX-II sequence from N. tabacum (tobacco) (13, 21). PCR amplification of cDNA from spinach produced a 565-bp product. The nucleotide sequence of the cDNA clone was highly homologous to tobacco PPX-II (67%). Full-length cDNA was obtained by 5' and 3'RACE PCR. The 1896-bp cDNA contained an open reading frame of 1593 bp, which encoded a 531-amino acid protein with a calculated molecular mass of 58,319 Da (Fig. 1). The deduced amino acid sequence shows a high degree of identity to tobacco mitochondrial Protox (70%), while the sequence identity to Protox I (plastidial Protox of spinach) (14) is relatively low (28%). The glycine-rich motif GXGXXG that had been previously proposed as a dinucleotide binding site of many flavin-containing proteins (25) was also found in the sequence (Fig. 1). All of the sequence data suggest that this cDNA encodes the spinach homolog of mitochondrial Protox, resulting in its designation as Protox II (gene name is SO-POX2).

N-terminal sequence alignment between Protox II and other tobacco PPX-II homologs (mitochondrial Protox isoforms) revealed the existence of 26- and 33-amino acid N-terminal extensions in the spinach and maize proteins, respectively (Fig. 2). Furthermore, the internal sequence following the second Met (Met-27 of spinach Protox II and Met-34 of maize Protox-2) aligns with N-terminal sequences of several plant mitochondrial Protoxes. The data were further digested with Ser and Thr but did not contain any acidic amino acids such as Asp or Glu. Such features are characteristic of chloroplast transit peptides (26). In fact, ChloroP, a program that searches for putative chloroplast targeting sequences (27), predicted that a chloroplast transit peptide is contained within both extension sequences (data not shown). On the other hand, a typical mi-
tochondrial import signal sequence was not found in either the extension or in the subsequent region following the second Met. It was reported that cDNA encoding tobacco mitochondrial Protox is able to complement the hemG mutation in E. coli (13). BT3 (DhemG::Kmr), a strain of E. coli defective in the hemG gene, grows very poorly even in rich media (14). To confirm the enzymatic activity of the cDNA gene products, two Protox II cDNAs of different lengths (Met-1 to Ile-531 and Met-27 to Ile-531) were ligated into vector pCR 2.1 in-frame with lacZ, and introduced into BT3 (DhemG::Kmr) E. coli cells. The mutation responsible for poor growth of the mutant was complemented by each cDNA, suggesting that both constructs have Protox activity.

To determine the number of copies of the Protox II gene in spinach, we performed Southern blot analysis of complete spinach genomic DNA digested with BglII or DraI, which have no recognition sites within Protox II gene, and EcoRI, which has one site within the gene. Under high stringency hybridization conditions, a single band in the BglII and DraI digests, and two bands in the EcoRI digest were observed, indicating that only one copy of the Protox II gene exists in the spinach genome (Fig. 3).

Localization of Spinach Protox II—To examine the localization of spinach Protox II, we prepared a polyclonal antibody against a recombinant fragment of spinach Protox II (Ser-101 to Ser-443) and purified it by affinity chromatography (see "Experimental Procedures"). The purified anti-Protox II antibody did not cross-react with recombinant spinach Protox I (data not shown). Immunoblot analysis with the antibody revealed the presence of two proteins with apparent molecular masses of 57 and 55 kDa in total spinach leaf extract (Fig. 4A, lane 1). When separate analyses of chloroplast and mitochondrial fractions were conducted, however, the 55-kDa band was found only in mitochondria, whereas the 57-kDa isoform was observed exclusively in chloroplasts (lanes 2 and 3). To identify more precisely the subcellular localization of these proteins, membrane fractions were prepared from mitochondria and chloroplasts, with further processing of the chloroplasts into thylakoid, envelope, and stroma fractions. Immunoblot analysis showed the presence of the 55-kDa protein in the mitochondrial membrane fraction and the 57-kDa protein in the chloroplast envelope fraction (Fig. 4B). These results suggest that Protox II exists as two isoforms with different molecular masses in spinach leaf and that the smaller and larger isoforms...
Fig. 3. Genomic DNA Southern blot analysis of Protox II in spinach. 20 μg of spinach genomic DNA was digested with restriction enzymes (B, BglII; D, DraiI; E, EcoRI) and subjected to Southern blot analysis. Hybridization was performed using full-length cDNA of spinach Protox II labeled nonradioactively by random priming. The sizes (in kb) of standard DNA fragments (1-kb ladder marker, Life Technologies, Inc.) are shown at left.

Fig. 4. Immunoblot analysis of Protox II in spinach leaf. Protein samples were separated by 10% (v/v) SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection was performed with a polyclonal antibody raised against recombinant Protox II of spinach. The positions of molecular mass markers (in kDa) are given at left. A, localization of Protox II in spinach leaf. Lane 1, total leaf extract (60 μg); lane 2, purified chloroplast fraction (20 μg); lane 3, mitochondrial fraction (10 μg). B, localization of Protox II within chloroplasts and mitochondria of spinach leaf. Lane 1, total purified chloroplast (20 μg); lane 2, stroma (10 μg); lane 3, envelope (1 μg); lane 4, thylakoids (10 μg); lane 5, mitochondrial membrane (10 μg).

are located on the mitochondrial membrane and chloroplast envelope membrane, respectively.

In Vitro Translation Products from Transcripts of Protox II Gene—Although immunoblot analysis revealed the presence of two Protox II isoforms in spinach (Fig. 4), Protox II gene exists as one copy gene in the spinach genome (Fig. 3). Moreover, spinach Protox II has an N-terminal extension composed of 26 amino acids, which precedes a region that is initiated by a second methionine (Met-27) and is highly identical to the N-terminal sequences of other plant mitochondrial Protox proteins (Fig. 2). The calculated molecular masses of Protox II with and without the extension (58,319 and 55,615 Da) correlate well to the observed masses of the two isoforms (57 and 55 kDa) observed in immunoblot analysis (Fig. 4). This correlation raises the possibility that the two isoforms were translated from the same spinach Protox II mRNA using two different in-frame AUG initiator codons. To explore this hypothesis, we analyzed the translation products from Protox II mRNA using in punctate organelles (Fig. 6B). The fluorescence pattern of an in vitro transcription/translation-coupled system from wheat germ lysate. When the pM1M2-POXII vector, containing the two putative in-frame initiation codons (Fig. 5A) was used as a template, two protein bands with apparent molecular masses of 59 and 55 kDa were detected by anti-Protox II antibody (Fig. 5B, lane 1). To confirm that these two proteins were translated separately from two in-frame initiation codons of one mRNA, two other vectors were constructed, pM1-POXII, in which the second ATG codon was mutated to ATC, and pM2-POXII, in which the first ATG codon was mutated to ATC (Fig. 5A). When the pM2-POXII vector was used as a template, only the 55-kDa protein band was detected (Fig. 5B, lane 2), whereas a 59-kDa protein was exclusively generated from pM1-POXII (lane 3). In a control experiment lacking plasmid, no protein was detected (data not shown). These results clearly indicate that two proteins of 59 and 55 kDa are translated from a single Protox II mRNA using two in-frame AUGs. The larger and smaller forms of Protox II were named Protox III and Protox IIS, respectively.

Transport of Protox III and Protox IIS into Chloroplasts and Mitochondria—In vivo transport of Protox III and Protox IIS was studied using green fluorescence protein (GFP). cDNA encoding the N-terminal region of Protox III (Met-1 to Thr-97) in which Met-27 was converted to Ile-27 (M1T97) and cDNA encoding the N-terminal region of Protox IIS (Met-27 to Thr-97) (M2T97) were each fused with the 5’ end of the GFP gene. Each construct was placed under the control of the cauliflower mosaic virus 35 S promoter. These plasmids were introduced into spinach leaves by bombardment, and transient expression was observed by fluorescence microscopy. When the Protox III construct, pM1T97-GFP, was introduced into a spinach leaf, green fluorescence was seen in chloroplasts (Fig. 6A). In contrast, in the spinach leaf transfected with pM2T97-GFP, the Protox IIS construct, the green fluorescence was observed only in punctate organelles (Fig. 6B). The fluorescence pattern of...
yeast CoxIV-GFP, which is known to be efficiently delivered into mitochondria (24) was very similar to that of pM27T97-GFP (Fig. 6C), indicating that M27T97-GFP protein does indeed co-localize with mitochondria in spinach. Transfection of control GFP, lacking an N-terminal tag, gave a diffuse pattern, with green fluorescence spread out over the guard cell (Fig. 6D).

Spatial Distribution of Protox II in Chloroplasts and Mitochondria—To identify the spatial distribution of Protox II within chloroplasts and mitochondria, we performed an immunogold electron microscopic analysis. When ultrathin sections of spinach leaf tissue were incubated with specific anti-Protox II antibody and gold-conjugated antiserum to rabbit immunoglobulin, gold particles were observed in chloroplasts and mitochondria. In chloroplasts, most of the gold particles were conjugated to the stromal side of the inner envelope membrane (Fig. 7A), whereas gold particles were found on the inner mitochondrial membrane (Fig. 7B). When preimmune serum was used, there was no appreciable binding of gold particles and the omission of primary antibody yielded negative results (data not shown). The observations suggest that Protox III associates with the stromal side of the inner envelope membrane, and that Protox II is found on the inner mitochondrial membrane.

Quantitation of Two Protox II Isoforms and Protox I in Chloroplasts and Mitochondria—The above results provide strong evidence that Protox II proteins are located not only in the mitochondria but also in chloroplasts. Spinach plastidal Protox, Protox I, associates primarily with the stromal side of the thylakoid membrane, with a small portion localizing to the stromal side of the inner envelope membrane (14). These results raise a new question as to how the two isoforms of Protox II and Protox I are distributed and how the function of each Protox is coordinated in spinach cells. To address the question, we performed quantitative antibody analysis of Protox III and Protox II. The level of Protox III in chloroplasts (1.2 ± 0.03 pmol/mg of chloroplast protein) was about 10 times lower than that of Protox I (14.7 ± 2.5 pmol/mg of chloroplast protein) (Table I). The amounts of Protox III and Protox I in the chloroplast envelope membrane are very similar (44.3 ± 7.8 and 48 ± 10.8 pmol/mg of envelope protein, respectively), whereas the amount of Protox I in thylakoid membrane was estimated at 28.4 ± 5.9 pmol/mg of thylakoid protein (Table I). Since almost half of all spinach chloroplast proteins exist in the thylakoid membrane and only 2% of total chloroplast proteins are present in the envelope membrane (28), the total amount of thylakoid-associated Protox I was estimated at about 14 pmol/mg of chloroplast protein, and the relative amount of Protox III and Protox I in the envelope membrane was estimated to be about 10 times less, at 1.2 pmol/mg of chloroplast protein. On the other hand, about 12.2 ± 0.1 pmol of Protox III/mg of mitochondria protein was estimated to exist in mitochondria. Since the ratio of chloroplast to mitochondrial proteins in one cell is not known, a precise ratio of chloroplast Protox to mitochondrial Protox in one cell could not be obtained. However, it is interesting that the amount of Protox IIS per mitochondria protein is similar to the amount of the two chloroplast Proteoxes per chloroplast protein (Table I).

**DISCUSSION**

**Alternative Translation Initiation at Two In-frame AUGs—**
Alternative transcription initiation and alternative translation initiation are well known mechanisms for dual protein production from a single gene (29). Some genes can produce more than one mRNA by use of alternative transcription initiation sites. Frequently, the production of multiple mRNAs has no functional consequence. However, if transcript heterogeneity leads to polypeptide heterogeneity, then it can result in the production of proteins with markedly different structures and properties (22, 29). The region upstream of the transcription start site often contains homopurine/homopyrimidine sequences, which are recognized by a protein factor and are able to initiate gene transcription (30, 31). Such a homopurine/homopyrimidine sequence was not found between the first and second ATGs of the Protox II genome DNA (data not shown). Moreover, only the longer of the cDNA variants, containing the first and second ATGs, was obtained in the process of Protox II cDNA cloning (Fig. 1), suggesting that the dual production of Protox III and IIS would not be a result of alternative transcription initiation.
Dual Targeting of Spinach Protoporphyrinogen Oxidase II

Another mechanism by which variability can be introduced into the N terminus of a polypeptide encoded by a single gene is alternative translation initiation from a single transcript using two potential in-frame translation initiation sites (29). The eukaryotic mechanism of translation initiation is distinctive in that the 40 S ribosomal subunit normally binds at the 5′ end of the mRNA. The small ribosomal subunit then migrates through the 5′-untranslated region until it encounters the first AUG codon. Flanking sequences modulate the efficiency with which the first AUG codon is recognized as a stop signal during the scanning phase of initiation. Initiation sites usually conform to all or part of a GCCRCC(GC)G sequence (32). The most highly conserved position within this consensus sequence is the purine at positions 3 and +4 (with a preference for G at +4 and no preference at −3). If a strong recognition sequence around the first AUG codon is absent, some small ribosomal subunits bypass the first AUG codon and initiate instead at a second or, rarely, even a third AUG. Consequently, such leaky scanning produces two or more proteins from one mRNA (32). Mutation analysis of the two putative AUG initiator codons indicated that initiation of translation of the two Protox II isoforms relies on both the first and second AUG codons in Protox II mRNA (Fig. 5). Moreover, analysis of the nucleotide sequence surrounding these two in-frame AUGs reveals that the second AUG has a stronger initiation recognition sequence (GTGATGGG) than the first (CGAATGGTA) (Fig. 1). These results strongly suggest that Protox IIL and IIS are translated from one mRNA using the first and second AUG codons and that the mechanism of the dual translation is leaky ribosomal scanning. It is still not known whether such leaky ribosomal scanning is controlled or uncontrolled. The in vitro translation system of Protox II must be useful to investigate the control mechanism of the leaky scanning.

Among other homologs of tobacco mitochondrial Protox, maize Protox-2 has a 33-amino acid N-terminal extension that is similar to that of Protox II (Fig. 2). Analysis of the upstream sequences of the first and second AUG codons showed that, as in spinach Protox, the second AUG also possesses a stronger initiator sequence than the first AUG, suggesting that the two isoforms may also be synthesized from a single maize Protox-2 mRNA. It is not known why only two Protoxes (spinach and maize) have N-terminal extensions. Several PPX-II homologs may actually have unknown N-terminal extensions, which would require more careful sequence analysis to identify.

**Intracellular Targeting of Two Protox II Isoforms**—GFP fused to the N-terminal region of spinach Protox III (pM1T97-GFP) was transported into chloroplasts, whereas GFP fused to the homologous region from Protox IIS (pM2T97) was targeted to mitochondria (Fig. 6). This implies that the extension may serve as a chloroplast transit sequence. The N-terminal transit peptide is usually cleaved off the precursor protein as it is transported across the two membranes of chloroplast (33). There was a small difference in the molecular sizes estimated by SDS-PAGE between the in vitro translation product (59 kDa) and mature protein identified in a chloroplast fraction (57 kDa). This size difference was also confirmed by co-electrophoresis of both proteins in the same lane (data not shown), suggesting that Protox IIS is processed after transport into the chloroplast.

A great majority of mitochondrial proteins is also encoded in the nuclear genome, synthesized in the cytosol, and then transported to the mitochondria. Most mitochondrial proteins are synthesized as larger precursors containing N-terminal cleavable extension peptides called presequences (34). However, MitoP, a commonly used computer program predicting mitochondrial presequences (35), predicted that spinach Protox IIS has no typical cleavable mitochondrial presequence. This prediction is in agreement with our experimental data that the molecular size of mature Protox IIS in leaves is identical with that of an in vitro synthesized product (Figs. 4 and 5B). Therefore, spinach Protox IIS would be put into the rare class of mitochondrial proteins lacking a cleavable presequence. The signal for mitochondrial targeting and import in such non-cleavable mitochondrial proteins is often still contained within the N-terminal region (36). In a transport experiment using a GFP fusion protein, it was revealed that a peptide consisting of residues from Met-27 to Thr-97 of Protox IIS is sufficient for mitochondrial transport (Fig. 6B). Such activity was not observed when only the first 23 amino acids (Met-27 to Val-49) were used for fusion with GFP (data not shown), suggesting that the information for mitochondrial targeting is contained over a wide area within the N terminus of Protox IIS.

Protein targeting to plant mitochondria and chloroplasts is usually very specific. Recently, the specificity of chloroplast and mitochondria targeting sequences has been studied using tandem fused proteins of both targeting sequences (37). The sequences coding for the presequence of the mitochondrial F1-ATPase β-subunit and the transit peptide of the chloroplast chlorophyll a/b-binding protein were fused in tandem and introduced into tobacco. When the mitochondrial presequence was inserted downstream to the chloroplast sequence, import into chloroplasts was observed. A mitochondrial presequence alone was able to direct transport to mitochondria; however, mitochondrial import of fusion proteins in which the chloroplast targeting sequence was linked downstream of the mitochondrial presequence was dramatically increased. These results indicate the importance of the more extreme N-terminal position of the targeting sequence in determining protein import specificity. The N terminus of the Protox IIL must exert a dominant influence, directing transport into chloroplasts, whereas its internal targeting sequence for mitochondrial transport may not be recognized by its cognate chaperone or receptor. The dual compartmentation of Protox II would thus be controlled in both translation and transport steps.

**Control of Chlorophyll and Heme Biosynthesis Pathways**—Two tetrapyrrole molecules, chlorophyll and heme, are synthesized in chloroplasts. A crucial branch point of the tetrapyrrole synthetic pathway in higher plants is the chelation of either Mg2+ to make chlorophyll or Fe2+ for heme catalyzed by magnesium chelatase or ferrochelatase, respectively (38). One model that has been proposed for the control of this branchpoint, based on biochemical studies, is that the two enzymes are spatially separated within the chloroplast, magnesium chelatase being associated with the envelope membrane (39), and

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**Table I**

Quantitation of the contents of three Protox isoforms in subcellular fractions

|                     | Chloroplasts | Envelope | Thylakoids | Mitochondria |
|---------------------|--------------|----------|------------|--------------|
|                      | pmol/mg cp protein | pmol/mg ev protein | pmol/mg thy protein | pmol/mg mt protein |
| Protox I             | 14.7 ± 2.5   | 44.3 ± 7.8 | 28.4 ± 5.9 | ND           |
| Protox IIL           | 1.2 ± 0.03   | 48.0 ± 10.8| ND         | ND           |
| Protox IIS           | ND           | ND       | ND         | 12.2 ± 0.1   |

The data are mean values of three experiments ± S.D. cp, chloroplast; ev, envelope; thy, thylakoids; mt, mitochondria; ND, not detected.
ferrochelatase existing exclusively in the thylakoids (40). However, recent studies using radiolabeled molecules have shown that ferrochelatase protein is located on both the thylakoid and envelope membranes, suggesting that heme biosynthesis occurs on the chloroplast envelope membrane and also that control of the branchpoint cannot be due to spatial separation of the two chelatases (41). Although this mechanism is far from being completely understood, our chloroplast Protox localization data present yet another attractive model for this branchpoint control. Spinach Protox I is preferentially associated with the stromal side of the thylakoid membrane, with a small portion of Protox I located on the stromal side of the inner envelope membrane (14). This implies that Protox I and Protox III are located at the same site of the chloroplast inner membrane. Protox must play the important role of supplying Proto IX to the magnesium chelatase and ferrochelatase in chloroplasts. It seems to be advantageous that Protox and chelatases are located close in space for proficient flow of tetrapyrrole synthesis. In fact, mammalian Protox interacts directly with ferrochelatase to facilitate the supply of Proto IX (15). The possibility exists, then, that each Protox isozyme (Protox I or Protox III) directly interacts with either magnesium chelatase or ferrochelatase and that the branchpoint is regulated by independently controlling the activity of each Protox isoenzyme. Evaluation of this hypothesis would depend on finding evidence of a direct, specific interaction between each Protox and a chelation enzyme.

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REFERENCES

1. Dailey, H. A. (1990) Biosynthesis of Heme and Chlorophylls, pp. 123–161, McGraw-Hill Publishing Co., New York
2. Dailey, H. A., and Karr, S. W. (1987) Biochemistry 26, 2067–2701
3. Camadro, J.-M., Thome, F., Brouillet, N., and Labbe, P. (1994) J. Biol. Chem. 269, 32085–32091
4. Siepker, L. J., Ford, M., de Kock, R., and Kramer, S. (1987) Biochim. Biophys. Acta 913, 349–358
5. Taketani, S., Yoshinaga, T., Furukawa, T., Kohno, H., Tekunaga, R., Nishimura, K., and Inokuchi, H. (1995) Eur. J. Biochem. 230, 760–765
6. Watanabe, N., Che, F.-S., Terashima, T., Takayama, S., Yoshida, S., and Isogai, A. (2000) Plant Cell Physiol. 41, 889–892
7. Sasarman, A., Letowski, J., Crauke, G., Ramirez, V., Nead, M. A., Jacobs, J. M., and Morais, R. (1993) Can. J. Microbiol. 39, 1155–1161
8. Hansson, M., and Hedestvedt, L. (1994) J. Bacteriol. 176, 5962–5970
9. Nishimura, K., Taketani, S., and Inokuchi, H. (1990) J. Biol. Chem. 265, 8076–8080
10. Dailey, T. A., Dailey, H. A., Meissner, P., and Prasad, A. R. K. (1995) Arch. Biochem. Biophys. 324, 379–384
11. Camadro, J.-M., and Labbe, P. (1996) J. Biol. Chem. 271, 9120–9126
12. Camadro, J.-M., Arnaud, S., Le Guen, L., Santos, R., Matringe, M., and Morne, R. (1999) in Peroxidizing Herbicides (Boger, P., and Wakabayashi, K., eds) pp. 245–277, Springer-Verlag, Berlin
13. Lermontova, I., Kruse, E., Mock, H.-P., and Grimm, B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8885–8900
14. Che, F.-S., Watanabe, N., Iwano, M., Inokuchi, H., Takayama, S., Yoshida, S., and Isogai, A. (2000) Plant Physiol. 124, 59–70
15. Ferreira, G. C., Andrew, T. L., Kair, S. W., and Dailey, H. A. (1988) J. Biol. Chem. 263, 3835–3839
16. Graser, E., Stj¨olring, S., Tanudji, M., and Whelan, J. (1998) Plant Mol. Biol. 36, 311–338
17. Volrath, S. L., Johnson, M. A., Potter, S. L., Ward, E. R., and Heifetz, P. B. (September 4, 1997) International Patent WO 97/22021
18. Duke, S. O., Lydon, J., Beecerril, J. M., Sherman, T. D., Lehnert, L. P., and Matsumoto, H. (1994) Am. Chem. Soc. Symp. Ser. 559, 191–204
19. Jacobs, J. M., and Jacobs, N. J. (1993) Plant Physiol. 101, 1181–1187
20. Ichinose, K., Akashi, K., and Mireau, H. (1996) Plant Mol. Biol. 30, 265–277