Sequences Required for the Activity of PTOX (IMMUTANS), a Plastid Terminal Oxidase

IN VITRO AND IN PLANTA MUTAGENESIS OF IRON-BINDING SITES AND A CONSERVED SEQUENCE THAT CORRESPONDS TO EXON 8*

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Aigen Fu, Sungsoon Park, and Steven Rodermel

From the Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa 50011

The thylakoid membranes of most photosynthetic organisms contain a terminal oxidase (PTOX, the product of the Arabidopsis IMMUTANS gene) that functions in the oxidation of the plastophysian pool. PTOX and AOX are diiron carboxylyte proteins, and based on crystal structures of other members of this protein class, a structural model of PTOX has been proposed in which the ligation sphere of the diiron center is composed of six conserved histidine and glutamate residues. We tested the functional significance of these residues by site-directed mutagenesis of PTOX in vitro and in planta, taking advantage null immutans alleles for the latter studies. These experiments showed that the six iron-binding sites do not tolerate change, even conservative ones. We also examined the significance of a conserved sequence in (or near) the PTOX active site that corresponds precisely to Exon 8 of the IM gene. In vitro and in planta mutagenesis revealed that conserved amino acids within this domain can be altered but that deletion of all or part of the domain abolishes activity. Because protein accumulates normally in the deletion mutants, the data suggest that the conformation of the Exon 8 sequence is important for PTOX activity. An allele of immutans (designated 3639) was identified that lacks the Exon 8 sequence; it does not accumulate PTOX protein. Chloroplast import assays revealed that mutant enzymes lacking Exon 8 have enhanced turnover. We conclude that the Exon 8 domain is required not only for PTOX activity but also for its stability.

The IMMUTANS (IM) gene of Arabidopsis thaliana codes for a plastid membrane protein that is distantly related to the alternative oxidase (AOX) of mitochondrial inner membranes (1, 2). AOX is a ubiquinol oxidase that catalyzes the four-electron reduction of oxygen to water and branches from the cytochrome pathway at the quinone pool (3, 4). AOX is found in all plants and in some fungi and protozoa, and it is thought to provide an overflow for carbon metabolism (3–5). Under stress conditions it might also play a role in minimizing the production of reactive oxygen species from electron transport (5–9).

Plants that lack IMMUTANS are variegated. Whereas cells in the green sectors of immutans (im) plants have normal-appearing chloroplasts, cells in the white sectors have vacuolated plastids that lack pigments and internal membrane structures. The white im sectors accumulate phytoene, a colorless C40 intermediate in carotenoid biosynthesis (10). This indicates that im is impaired in the activity of phytoene desaturase, which converts phytoene to δ-carotene. Because of its resemblance to AOX, it was early hypothesized that IM is a plastid quinol oxidase that functions as a redox component in phytoene desaturation. According to this hypothesis, electrons are transferred from phytoene to plastophysian (via phytoene desaturase) and then from plastophysian to oxygen (via IM) (1, 2). It was further suggested that a lack of IM (as in im) results in overreduction of the plastophysian pool and that this is responsible for the build-up of phytoene in the membranes. As a consequence, the production of downstream, photoprotective (colored) carotenoids would be impaired, and under high light illumination conditions, photooxidized plastids would be generated. These would give rise to white cells and sectors as the leaf develops. In support of this hypothesis, IM has quinol oxidase activity in vitro and in vivo (11–14).

IM is expressed in nearly all Arabidopsis tissues and organs (15). Consistent with the idea that IM plays an important role in plastid metabolism, the differentiation of many plastid and tissue types is impaired in im (15), as well as in the ghost variegation mutant of tomato (16). GHOST is the tomato ortholog of IM (12, 16). The ubiquitous expression of IM suggests that its function might not be limited to carotenogenesis. In support of this notion, evidence has accumulated that IM is the elusive terminal oxidase of chlororespiration (and hence is frequently designated “PTOX,” for plastid terminal oxidase) (11, 13). In addition, IM has been implicated as an important component in the arsenal of plastid responses to oxidative stress, likely as a “safety valve” for the dissipation of excess electron flow (17–20).

Our current working hypothesis is that IM is a versatile alternative electron sink in plastid membranes and that it lies at the intersection of many redox pathways.

Sequence comparisons have revealed that AOX and PTOX are non-heme diiron carboxylyte proteins (21–25). By analogy to crystal structure determinations of non-plant members of this protein class, it has been proposed that the diiron centers of AOX and PTOX are coordinated by four carboxylate and two histidine residues on a four helix bundle (Fig. 1, A and 1B) (23–25). Support for this model has come from EPR spectroscopy of AOX, as well as from mutagenesis of five of the six proposed iron ligands of AOX using prokaryotic model systems to test function (such as a heme-deficient Escherichia coli strain) (3, 26–30). In addition to the four alpha helices that bear the active site, AOX and PTOX have a fifth, smaller α-helix. This helix and part of helix 1 are thought to be embedded in the membrane but not span it, i.e. AOX and PTOX are modeled as interfacial membrane proteins (Fig. 1B) (23–25).

The proposition that the structure of PTOX is similar to that of AOX is based on a single sequence, IM from A. thaliana (1, 2). The first purpose of this paper was to test the universality of the Berthold/
Sequences Required for PTOX Activity

**TABLE ONE**

| Mutation   | Primer sequences |
|------------|------------------|
| E136A      | 5'-gccgactctatgctgactgtccc-3' |
| E136D      | 5'-gccgactctatgctgactgtccc-3' |
| E136H      | 5'-gccgactctatgctgactgtccc-3' |
| E175A      | 5'-ctacttttcgggacctgatgtccc-3' |
| E175D      | 5'-ctacttttcgggacctgatgtccc-3' |
| H177A      | 5'-ctacttttcgggacctgatgtccc-3' |
| H178A      | 5'-ctacttttcgggacctgatgtccc-3' |
| H178E      | 5'-ctacttttcgggacctgatgtccc-3' |
| H178N      | 5'-ctacttttcgggacctgatgtccc-3' |
| E227A      | 5'-ctacttttcgggacctgatgtccc-3' |
| E227D      | 5'-ctacttttcgggacctgatgtccc-3' |
| E227H      | 5'-ctacttttcgggacctgatgtccc-3' |
| E296A      | 5'-ctacttttcgggacctgatgtccc-3' |
| E296D      | 5'-ctacttttcgggacctgatgtccc-3' |
| E296H      | 5'-ctacttttcgggacctgatgtccc-3' |
| E298A      | 5'-ctacttttcgggacctgatgtccc-3' |
| H299A      | 5'-ctacttttcgggacctgatgtccc-3' |
| H299E      | 5'-ctacttttcgggacctgatgtccc-3' |
| H299N      | 5'-ctacttttcgggacctgatgtccc-3' |

**TABLE TWO**

| Mutation   | Primer sequences |
|------------|------------------|
| DEX8       | 5'-gccgactctatgctgactgtccc-3' |
| D1–8       | 5'-gccgactctatgctgactgtccc-3' |
| D9–16      | 5'-gccgactctatgctgactgtccc-3' |
| D267A      | 5'-gccgactctatgctgactgtccc-3' |
| E268A      | 5'-gccgactctatgctgactgtccc-3' |
| E269A      | 5'-gccgactctatgctgactgtccc-3' |
| Q270A      | 5'-gccgactctatgctgactgtccc-3' |
| T271A      | 5'-gccgactctatgctgactgtccc-3' |
| R278A      | 5'-gccgactctatgctgactgtccc-3' |
| R279A      | 5'-gccgactctatgctgactgtccc-3' |
| P280A      | 5'-gccgactctatgctgactgtccc-3' |

Andersson/Nordlund model of PTOX (24), given the diversity of PTOX sequences that have become available since the model was proposed. These studies revealed that 1) the six iron ligands are perfectly conserved in PTOX from diverse sources; and 2) nearly all PTOX enzymes have a conserved 16 amino acid sequence that is not present in AOX, and which corresponds precisely to Exon 8 of the higher plant gene. Thus, the second purpose of this paper was to examine the functional significance of the putative iron ligands and Exon 8 sequences both in vitro and in planta. The in planta experiments were made possible by the availability of null im alleles of Arabidopsis and by the ability to obtain fully viable transgenic im plants that bear constructs containing mutated IM genes. Finally, we identified an Arabidopsis im allele that lacks Exon 8 and used this mutant as a tool to further assess the importance of the Exon 8 sequence for PTOX structure and function.

**MATERIALS AND METHODS**

Plant Strains—Strain 3639 is an uncharacterized *A. thaliana* variegation mutant from the Arabidopsis Biological Resource Center (Ohio State University). It is light-sensitive and was maintained under conditions previously described for other light-sensitive Arabidopsis variegations (10). The *spotty* allele of im (10) and 3639 were used in this report; both are in the Columbia background. Wild type Columbia seedlings served as “wild type” controls.

In Vitro Site-directed Mutagenesis—A full-length IM cDNA has previously been isolated in our laboratory (1). A truncated version of this cDNA that lacks the chloroplast transit peptide sequence was generated by PCR amplification, and the resulting sequence was confirmed by DNA sequencing. The truncated cDNA was cloned into the BamHI and Nhel sites of pET-11a (Novagen, Madison, WI), and the QuikChange™ Site-Directed Mutagenesis protocol was used to generate mutations (Stratagene, La Jolla, CA). Instructions provided by the manufacturer were followed, except for deletion mutations, where a 48 °C annealing temperature was used instead of 55 °C. All the mutated sequences were confirmed by sequencing. Following standard notation, the mutant clones are designated by the amino acid that was altered, its location in the IM sequence, and the amino acid to which it was changed (e.g. H299A: His was changed to Ala).

The primers listed in TABLES ONE and TWO were used to produce mutations in the iron-binding sites of IM and in the Exon 8 sequence.
DEX8 is a deletion of the entire Exon 8 sequence, while D1–8 and D9–16 are deletions of the first eight or last eight amino acids of Exon 8.

Site-directed Mutagenesis in Plants—For in planta mutagenesis experiments, mutations were generated using the same procedures and primers described above for the in vitro experiments, except that the full-length IM cDNA was used (to assure proper chloroplast targeting) (1). The mutant DNAs were cloned behind the cauliflower mosaic virus (CaMV) 35S promoter in the binary vector pBluescript II and the constructs were transferred into Agrobacterium tumefaciens; the floral dip method (31) was used to transform im plants (spotty allele). Kanamycin-resistant seedlings were selected at the T₁ generation on plates containing 1X Murashige-Skoog salts (pH 5.7), 1% sucrose and 50 μg/ml kanamycin. PCR and Southern Blotting methods were used to verify that the plants were transformed. Phenotypic analyses were performed on T₂ generation plants.

In Vitro PTOX Activity Assays—PTOX activity assays were conducted in vitro using procedures described by Josse et al. (12, 14). In brief, the mutant constructs were transformed into E. coli strain BL21-DE3, and the cells were grown in LB medium supplemented with 100 μg/ml ampicillin until they reached an A600 of 0.6. Protein expression was induced by the addition of 1.6 mM isopropyl-1-thio-β-D-galactosynaroside (final concentration). After 2 h, the cells were harvested by centrifugation (3000 × g for 10 min at 4 °C), and the pellet was resuspended on ice in a lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, and 0.3% protease inhibitor mixture (Sigma). The resuspension was sonicated briefly and unbroken cells were removed by centrifugation (3000 × g for 10 min at 4 °C). The supernatant was then centrifuged in a Beckman L8–70W ultracentrifuge (200,000 × g for 2 h at 4 °C), and the membrane pellet was resuspended in a solution containing 0.75 μM sucrose, 0.2 mM Tris-HCl (pH 7.5). The concentration of proteins in the pellet was measured using the Bio-Rad protocol with bovine serum albumin as a standard.

Detection of RNA and Protein—Procedures for total cell RNA isolation and Northern blotting have been described (32). The nitrocellulose filters were probed with labeled IM sequences, and RNAs were visualized by phosphorimaging analysis.

E. coli proteins were isolated as described above. To isolate partially purified chloroplasts, green leaf tissues were collected from Arabidopsis seedlings (3–4 weeks old) and homogenized in a Waring blender in the cold in a solution containing 0.33 M sorbitol, 10 mM EDTA, and 50 mM HEPES (pH 8.0). The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 2,600 g for 10 min. The pellet was washed with 10 mM MOPS (pH 8.0). Following centrifugation (10,000 × g for 10 min), the pellet was resuspended in 0.33 M sorbitol, 5 mM MgCl₂, 50 mM HEPES (pH 8.0). Chlorophyll concentrations were measured on the resuspended membranes as outlined previously (32).

Procedures for Western blotting have been described (32). In brief, equal amounts of chlorophyll (5 μg) were electrophoresed through 12.5% SDS-polyacrylamide gels, and the proteins were transferred to a nitrocellulose filter. The filter was incubated with a polyclonal antibody generated to the Arabidopsis IM protein (1:3,000 dilution) (17), and the proteins were visualized using the ECL immunodetection procedure (Pierce, Erembodegem, Belgium).

Protein Import Assays—An IM cDNA containing the entire coding sequence and an IM cDNA in which Exon 8 was deleted (DEX8) (see above) were cloned into the BamHI and Nhel sites of pET-11a. These two constructs were used as DNA templates to carry out in vitro transcription and translation with the TNT Quick Coupled Transcription/Translation system (Promega, Madison, WI). A standard reaction mixture of 50 μl contained 40 μl of TNT Quick Master, 20 μCi of [35S]methionine, and 1 μg of template DNA.

The import assays were conducted with intact pea chloroplasts using established methods (32, 33, 34). In brief, leaf tissues from pea seedlings (10–15 days-old) were homogenized in a blender (in the cold) in GR buffer: 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 0.5g/liter bovine serum albumin, 5 mM ascorbate, and 50 mM HEPES-KOH (pH 8.0). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged 5 min at 2,600 × g. The resulting crude chloroplast pellet was resuspended in GR buffer, then loaded onto a Percoll gradient. After centrifuging 10 min at 10,500 × g, intact chloroplasts were removed and washed twice in a buffer containing 0.33 M sorbitol and 50 mM HEPES-KOH (pH 8.0).

A standard import reaction of 500 μl contained 330 mM sorbitol, 50 mM HEPES-KOH (pH 8.0), 10 mM NaHCO₃, 8 mM MgCl₂, 0.1% bovine serum albumin, 1.5 mM dithiothreitol, 1.2 mg antipain (Sigma), 5 mM ATP and 40 μl of 35S-labeled IM transcription/translation products. The import assay was initiated by adding chloroplasts (corresponding to 200 μg of chlorophyll). The preparations were maintained at 25 °C in the light. After 30 min, most of the label had been incorporated into the chloroplasts. The fate of the labeled IM protein was monitored for up to 3 h.

RESULTS AND DISCUSSION

Mutagenesis of Putative Iron-binding Ligands in Vitro—Berthold and colleagues have proposed that the structure of PTOX is similar to that of AOX (Fig. 1) (24). This hypothesis is based on several dozen AOX sequences, but a single PTOX sequence, IM from Arabidopsis (1, 2). As a first approach to test the validity of this model, we compared PTOX sequences that have become available since the model was proposed. Fig. 1C shows the phylogenetic relatedness of five higher plant, two green algal and five cyanobacterial sequences, using Arabidopsis AOX1a as an outgroup. The long branch length indicates that AOX is distantly related to PTOX, as reported previously (1). The higher plant PTOX enzymes form a distinct clade that can be separated into monocot (rice and wheat) and dicot (Arabidopsis, pepper, and tomato) proteins. The green algal sequences (Bigelowiella and Chlamydomonas) also cluster together. Sequence analyses of completed genomes and/or molecular hybridization experiments have revealed that PTOX is a single copy gene in Arabidopsis, tomato, and rice (1, 12, 16, 35).

Fig. 1A shows that PTOX proteins, regardless of source, resemble Arabidopsis IM in that their active sites are contained within a four helix bundle. They also have a fifth, shorter α-helix that likely anchors PTOX in the plastid membrane (24). We also found that all PTOX enzymes have six perfectly conserved amino acids that could potentially function as iron-binding ligands. These residues are glutamate and histidine; in both PTOX and AOX there are two perfectly conserved EXXH sequences and two perfectly conserved glutamates in the active site region (Fig. 1A). Taken together, these data support the Berthold/Andersson/Nordlund model of PTOX (24).
Sequences Required for PTOX Activity

FIGURE 1. Structures of AOX and PTOX. A, AOX and PTOX have four helices that contain six putative iron ligands in the dimer center at the active site (21–23). The four conserved helices with their Glu and His residues are in gray; the dimerization domain of AOX with its conserved regulatory Cys is cross-hatched; and the Exon 8 insertion (EX-8) in PTOX is in black. "TP" are organelle targeting sequences ("transit peptides"). Numbering is according to that of the AOX1a and PTOX proteins from Arabidopsis. B, PTOX is proposed to be an interfacial membrane protein with a diiron center composed of two EXH motifs on helices 2 and 4 (oriented anti-parallel to each other), and two Glu residues on helices 1 and 3 (also oriented anti-parallel to each other) (22). The Exon 8 insertion is indicated by a bold line. C, the higher plant sequences are from A. thaliana (At IM, gi 4138855), Capsicum annuum (pepper Pftf, gi 9937103), Lycopersicon esculentum (tomato GH5, gi 9937101), Oryza sativa (rice IM, gi 21105122), and Triticum aestivum (wheat IM, gi 9837152). The algal sequences are from Bifidovolvia natans (gi 32307546) and Chlamydomonas reinhardti (gi 201492540), and the cyanobacterial ones are from Gloeobacter violaceus PCC7421 (gi 35211763), Synechococcus sp. WH8102 (gi 3365421), Anabaena variabilis sp. ATCC29413 (gi, 4550896), Prochlorococcus marinus MED4 (gi, 3360894), and Anabaena variabilis sp. PCC7120 (gi, 17229588). Full-length amino acid sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw/). A. thaliana Aox1a (AtAOX1a, gi 3915639) served as an outgroup. Included are all PTOX homologs in the GenBank non-redundant protein database as of July 1, 2005.

As a first approach to test the functional significance of the six putative iron ligands of PTOX, we conducted site-directed mutagenesis experiments using an in vitro assay activity developed by Josse et al. (12, 14). In this assay, O2 consumption is measured in membranes isolated from E. coli that have been transformed with various mutant IM sequences. Addition of NADH as an electron donor results in the formation of reduced quinone (by membrane-bound NADH dehydrogenase), and electrons are then transferred to molecular oxygen via PTOX or the cytochrome pathway. PTOX activity is inhibited by pyrogallol analogues, such as propyl gallate and octyl gallate, but is insensitive to cyanide (12, 14). Thus, O2 consumption occurs by the cytochrome pathway in the absence of KCN but by PTOX activity in the presence of KCN. KCN and n-propyl gallate (n-PG) together abolish O2 consumption. PTOX becomes engaged in this system only when the cytochrome pathway is blocked (12, 14).

For our experiments, the conserved iron-binding ligands of Arabidopsis PTOX (IM gene product) were changed to conservative or non-conservative amino acids; the Glu residues were changed to Ala (non-conservative) or Asp (conservative), and the His residues were changed to Ala (non-conservative), or Asn (conservative with respect to the space occupied, but non-conservative with respect to charge). Glu was also changed to Asp (or vice versa) to test whether one type of iron ligand can substitute for another. As controls, His or Glu residues that reside near the putative iron-binding sites were mutated to Ala. The constructs were transformed into E. coli, membranes were isolated, and respiratory measurements were performed in the presence of KCN and n-PG.

Fig. 2 shows the O2 consumption traces of four representative experiments to test whether His178 (in helix 2) is essential for PTOX activity. In contrast to membranes from non-transformed E. coli (Fig. 2A and Fig. 3A, lane 1), membranes from cells that contain wild type PTOX display cyanide-resistant O2 consumption (Fig. 2B and Fig. 3, lane 2). This activity is inhibited by n-PG, suggesting that it arose from PTOX. Fig. 2D (and Fig. 3A, lane 10) reveals that mutation of His178 to Ala (H178A) nearly abolishes cyanide-resistant O2 consumption but that mutation of the adjoining residue to Ala (H177A) has no effect on consumption (Fig. 2C and Fig. 3A, lane 9). n-PG sensitive O2 consumption is also blocked by a partially conservative substitution of His178 to Asn (H178N) (Fig. 3, lane 12), as well as by alteration of His to Glu (H178E) (Fig. 3, lane 11). Taken together, these data indicate that His178 is required for PTOX activity and/or stability and that His at this site is essential.

Results similar to those in Fig. 2 were obtained for each of the other five putative iron ligands (Fig. 3A). 1) O2 consumption is blocked by non-conservative substitutions (E136A, E175A, E227A, E296A, and H299A) (lanes 3, 6, 13, 16, and 20). 2) O2 consumption is inhibited by conservative substitutions of Glu to Asp (E136D, E175D, E227D, and E296D) (lanes 4, 7, 14, and 17). 3) The iron ligands do not appear to be interconvertible, i.e. O2 consumption is inhibited when Glu residues are converted to Asp, or vice versa (E136H, E175H, E227H, E296H, and H299E) (lanes 5, 8, 15, 18, and 21). 4) O2 consumption is blocked by a
partially conservative substitution of His to Asn (H299N) (lane 22), similar to H178N noted above (lane 12). 5) Mutations at carbohydrate residues that reside near the iron ligands have little, if any, affect on O2 consumption (E298A, lane 19), and equal amounts were electrophoresed through 12.5% SDS-PAGE gels, then transferred to nitrocellulose membranes. The membranes were treated with an antibody to PTOX and visualized by the ECL system. Lanes are as in A, C, the same mutations in as in the in vitro mutagenesis experiments were introduced into im plants. Kanamycin-resistant seedlings were scored by PCR and Southern blotting for the presence of the kanamycin gene (NPTII). Over 100 bone fide transformation events were examined (at least eight per construct); the figure shows 10 representative T2 generation seedlings (10 different constructs). All plants were grown for 3-4 weeks under continuous light (60-90 μmol m-2 s-1). D, Western immunoblot analyses were conducted using 5 μg of chlorophyll per lane from chloroplast membranes of partially purified plastids using an antibody to Arabidopsis PTOX.

FIGURE 3. Mutagenesis of active site iron-binding ligands in vitro and in planta. A, E. coli oxygen consumption rates were determined from the slopes of O2 traces following the addition of 1 μM NADH, 3 μM KCN, and 0.5 μM n-PG as shown in Fig. 2. The PTOX activity is represented by the oxygen consumption rates in the presence of 3 μM KCN minus the oxygen consumption rates in the presence of 0.5 μM n-PG. The rates are the average values of four measurements (± S.D.). Lane 1, E. coli with empty pET11a vector; lane 2, E. coli with the pET11a/PTOX (wild type). Lanes 3–22, E. coli containing the expression vector with the following mutations: E136A (lane 3), E136D (lane 4), E136H (lane 5), E175A (lane 6), E175D (lane 7), E175H (lane 8), H177A (lane 9), H178A (lane 10), H178E (lane 11), E178N (lane 12), E227A (lane 13), E227D (lane 14), E227H (lane 15), E296A (lane 16), E296D (lane 17), E296H (lane 18), E296I (lane 19), H299A (lane 20), H299E (lane 21), and H299N (lane 22). B, total membrane proteins were isolated from transformed E. coli, and the protein content was determined by the Lowry method. C, Western blot analysis using the antibodies against PTOX in lane 7, E136A; lane 9, H178A; lane 12, E227A; lane 16, E296A; and lane 22, H299N (lanes 3–22). D, Western immunoblot analyses were conducted using 5 μg of chlorophyll per lane from chloroplast membranes of partially purified plastids using an antibody to Arabidopsis PTOX.

FIGURE 4. PTOX Exon 8 sequences from diverse species. Exon 8 sequences (48 bp) were aligned from the same species as in Fig. 2. The dash denotes a gap in the sequence. Degree of conservation (”Consensus”) correlates with font size.

Yet, it is still possible that they fail to fold properly in the membrane. We cannot rule this out. However, several lines of evidence make this possibility unlikely. First, it has been reported that the three-dimensional structures of diiron carboxylate proteins are quite stable and that the metal ligands contribute little to the conformation of the active site (36, 37). The overall conformation of these proteins also appears to be resistant to alterations in charge and mobility of side chains in the vicinity of the diiron center. Our data support this idea inasmuch as PTOX enzymes with mutations at sites near the putative iron-binding ligands have wild type levels of cyanide-resistant O2 consumption.

Mutagenesis of Putative Iron-Binding Ligands in Planta—To examine the impact of each of the mutations described above on PTOX activity in planta, the mutant constructs were introduced into a null im allele (spotty) (1). Full-length cDNAs were used for proper chloroplast targeting, and the cDNAs were cloned behind the CaMV 35S promoter; phenotypes were examined in the T2 plants. At least eight independent transformants were examined per construct (a minimum of 168 different transformation events for the 21 mutations in Fig. 3A). The data from 10 representative transformants (10 different constructs) are shown in Fig. 3C. These data show that: 1) transformation of im with a wild type IM cDNA (“WT” lane) abolishes the variegation phenotype, generating normal appearing plants. This demonstrates that the IM cDNA is able to complement the im defect. 2) Control transformations of im with the empty binary vector (“pBI121” lane) remain variegated. 3) T2 plants from the H177A and E298A transformations (i.e. mutant DNAs with changes at nearby carbohydrate residues) resemble wild type, suggesting that these two mutations do not impact PTOX activity in planta. 4) T2 plants from the E136A, E175A, H178A, E227A, E296A, and H299A transformations remain variegated, suggesting that these mutations affect PTOX activity in planta. Similar results were obtained for the 12 other mutations: E136D, E136H, E175D, E175H, H178N, H178E, E227D, E227H, E296D, E296H, H299E, and H299N (data not shown).

Fig. 3D shows that the mutants in Fig. 3C have similar levels of IM protein accumulation. This suggests that a failure to complement im is not due to a lack of IM expression and/or protein abundance. Therefore, we hypothesize that conservative and non-conservative mutations in any of the six putative iron-binding sites are deleterious to PTOX activity in planta. The only two mutations that do not seem to affect activity are those in carbohydrate residues that reside near the iron-binding ligands.

In summary, we conclude that the results from the in vitro and in planta experiments are in striking agreement with one another and show that the six putative iron ligands of PTOX are required for enzyme activity. In addition, these residues do not tolerate change, even con-
confirmed by Albury of the Sauromatum guttatum mutation does not affect protein stability. Of the six sites, Berthold et al. demonstrated directly that two of them bind iron (Glu222 and H327A) do not restore aerobic respiration to a heme-deficient strain Arabidopsis AOX1a iron-binding sites (E222A, H225A, E273A, and H327A) do not restore aerobic respiration to a heme-deficient strain of *E. coli*. However, the lack of complementation was due to protein instability in the H225A and H327A mutants, rather than to an inactive enzyme, as in the other two cases (monitored by lack of detectable mixed valent EPR signals characteristic of a binuclear iron center). The identifications of Glu222 as important for activity has been confirmed by Albury et al. (30), who reported that an E217A mutant of the *Sauromatum guttatum* AOX (analogous to Glu222 of Arabidopsis AOX1a) is inactive in an *S. pombe* expression system; this mutation does not affect protein stability. Of the six sites, Berthold et al. (26) demonstrated directly that two of them bind iron (Glu222 and Glu273, corresponding to Glu175 and Glu227 of PTOX). Although physical evidence that PTOX binds iron is lacking, Josse et al. (14) found that PTOX requires iron for activity.

Structural Domains of PTOX Essential for Activity: Exon 8—Umbach and Siedow (38) hypothesized that there are two general types of AOX, higher plant and fungal, distinguished by their subunit structures and regulators: α-keto acid or succinate stimulation of activity corresponds with a dimeric structure, while purine nucleotide stimulation corresponds with a monomeric structure. The N terminus of AOX contains a conserved cysteine (Cys227 of Arabidopsis AOX1a, see Fig. 1A) that is responsible for homodimer formation via the formation of a disulfide bridge (3–5, 38). Activation of the enzyme occurs by reduction of this linkage and the binding of pyruvate (and other organic acids) to the free Cys residue (39, 40). This Cys is embedded within a conserved ~40-amino acid domain that might mediate protein/protein interactions (“dimerization domain,” Fig. 1A). Whereas some species (e.g. rice and tomato) lack the regulatory Cys and instead have a serine at this site (and are activated by succinate), these enzymes nonetheless form dimers, perhaps via their dimerization domains (38). In contrast to the higher plant enzymes, fungal AOX proteins lack the regulatory Cys, as well as the dimerization domain (38). Yet, they contain a conserved ~20–25 amino acid sequence in their N terminus that is not present in the higher plant enzymes; the function of this domain is obscure.

FIGURE 5. Mutagenesis of PTOX Exon 8 sequences *in vitro* and *in planta*. A, the figure legend is the same as for Fig. 3A. The rates are the average values of four measurements. B, the figure legend is the same as for Fig. 3B. C, the figure legend is the same as for Fig. 3C. D, the figure legend is the same as for Fig. 3D.

### FIGURE 6. Wild type and immutans (3639 allele) plants. A, mutant strain 3639 was obtained from the Arabidopis Biological Resource Center and is an allele of *immutans* that lacks Exon 8. B, Northern analyses were conducted using 10 μg of total cell mRNAs from WT leaves and from green sectors of 3639. The nitrocellulose filters were probed with an *E. coli* cDNA (B). C, Western immunoblot analyses were conducted using 5 μg of chlorophyll per lane of partially purified chloroplast membranes from WT leaves and from green sectors of 3639. The filters were probed with an antibody to Arabidopsis PTOX.

To test whether the Exon 8 domain affects PTOX activity, three deletion constructs were generated: DEX8, which lacks the entire Exon 8 sequence; D1–8, in which the first eight amino acids of Exon 8 were deleted, and D9–16, in which the last eight amino acids of Exon 8 were deleted. Point mutations were also generated to the seven perfectly conserved amino acids in Exon 8 (D267A, E268A, F269A, Q270A, T271A, R278A R279A, and P280A), as well as to the highly conserved E268 (E268A). The mutants were then tested in *E. coli* for their cyanide-resistant, α-PG-sensitive O2 consumption activity (Figs. 2 and 3A). They were also introduced into *in* to test their *in planta* activity (Fig. 3C).

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Fig. 5C shows that mutations in the eight conserved amino acids do not impact PTOX activity in planta as much as the T_{2} plants resemble wild type. In contrast, DEX8, D1–8, and D9–16 T_{2} plants are variegated. This suggests that the deleted sequences are essential for activity. The various transformants in Fig. 5C have wild type levels of IM protein, and the deletion mutant proteins are smaller than normal, again as anticipated (Fig. 5D). Considered together, the in vitro and in planta data are in accord with one another.

In an ongoing survey of variegation mutants of Arabidopsis, we discovered one that turned out to be allelic to im (strain 3639, obtained from the Ohio State Arabidopsis Biological Resource Center) and that lacks Exon 8. This allele was generated by ethyl methane sulfonate and purified pea chloroplasts. Other investigators have shown that import is nearly complete 30 min after mixing the plastids and labeled precursor proteins together (33, 34). This is illustrated in Fig. 7A, which shows substantial import of the wild type and mutant proteins after 30-min incubation (time “0”): the upper band is the precursor protein, and the lower band is the imported, mature protein. As in Fig. 5D, the DEX8 proteins are smaller than normal because of the Exon 8 deletion. 30 min after incubation, the plastids were washed, and the fate of the labeled proteins was followed during a 3-h chase. Fig. 7B reveals that both proteins decrease in amount during the chase, and that after 3 h, the levels of the wild type protein decrease about 20% versus 60% for the mutant protein (Fig. 7B). This suggests that the DEX8 protein is less stable than the wild type protein. We conclude that deletion of the Exon 8 domain causes PTOX to be turned over more rapidly than normal in strain 3639. This suggests that in addition to being important for activity, the Exon 8 domain is essential for PTOX stability, e.g., the protein might be turned over if this domain is necessary for assembly with other proteins in the membrane.

Why does the mutant PTOX behave differently in 3639 versus the DEX8 overexpressor? One difference between the two strains is the promoter used to drive expression of the Exon 8 deletion: in 3639, transcription is driven by the relatively weak IM promoter, while in the DEX8 plants, transcription is driven by the high level expression CaMV 35S promoter. This promoter difference is reflected in the abundance of IM mRNAs, which are significantly higher in the DEX8 plants (data not shown). Therefore, we speculate that a post-transcriptional system is limiting in the DEX8 plants and, consequently, that this causes DEX8 proteins to accumulate. One possibility is that the capacity of the proteolytic system(s) of the plastid responsible for degrading defective or excess PTOX is limiting and not sufficient to handle the large amounts of protein that are produced.

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