The mechanism of Arabidopsis immutans variegation

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The mechanism of Arabidopsis \textit{immutans} variegation

by

Aigen Fu

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ABSTRACT

The Arabidopsis \textit{immutans} (\textit{im}) is variegated with green sectors and white sectors containing defective plastids due to a nuclear gene mutation. IM is a plastid terminal oxidase (PTOX) sharing similarity with mitochondrial alternative oxidase (AOX). In order to better understand IM’s biological roles and variegation mechanism of \textit{im}, I characterized \textit{im} plants at different levels.

A structural model of IM, in which the dirron reaction center is composed of two conserved histidine and four glutamate residues, was tested by mutagenesis \textit{in vitro} and \textit{in planta}. It that these six conserved residues were found essential for IM activity and do not tolerate changes. Mutagenesis screening of 14 other conserved residues showed that two residues are essential, and four are important but not essential for IM activity. A 16 aa sequence uniquely present in PTOX was also found to be required for PTOX activity and stability. Overexpression of AOX in chloroplasts functionally rescues the variegation phenotype of \textit{im}. And AOX deleted of the dimerization domain is more efficient to compensate \textit{im} variegation phenotype, while addition of \textit{IM} exon-8 sequence costs AOX function in chloroplasts.

A proteomic analysis suggested plastids in \textit{im} white sectors were alive even without photosynthetic capacity. Defective plastids caused by photodamage induced a higher respiratory activity and various upregulated cytosolic proteins. Downregulated FtsZ1 level and microscopy analysis suggested that the division of photooxidized plastids were suppressed. Downregualtion of IM to \textasciitilde1\% of wild type levels did not compromise plant growth suggests that IM is normally in excess. Phenotypical observations indicated that IM
does not significantly affect proplastids or differentiated plastids, but plays an essential role in early plastid differentiation stages.

A hypothesis was proposed to interpret *im* variegation mechanism. Proplastids develop into defective plastids due to photodamage without carotenoid protection; while some differentiating plastids escape photodamage and form chloroplasts due to shade effects. In a differentiating heteroplastidic cell, division of defective plastids was suppressed while chloroplasts maintain post-mitotic division. Consequently, homoplastidic and heteroplastidic differentiating cells with normal chloroplasts lead to green sector formation, while homoplastidic cells with defective plastids lead to white sector formation.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation consists of six chapters, including one published paper and three manuscripts. Chapter one is a general introduction that reviews background knowledge related to the studies performed in this thesis. Chapter two is a paper published in J. Biol. Chem. (Fu et al., 2005), which covers mutagenesis analysis of iron-binding sites in the PTOX and AOX reaction center and a 16 amino acid fragment that is unique to PTOX versus AOX sequences. Chapter three reports mutagenesis studies on the role of 14 other conserved residues of the AOX and PTOX active sites. Chapter four reports a proteomics analysis of leaf proteins from wild type plants, green tissues, and white tissues of im mutant plants. Chapter five demonstrates that overexpression of mitochondrial AOX in chloroplasts is able to compensate for the variegation phenotype of Arabidopsis im mutant plants. Chapter Six briefly summarizes all the work in this thesis.

Literature Review

Plant plastids

One of the distinguishing characteristics of plant cells is the presence of the plastid, a double membrane-enclosed organelle. In addition to photosynthesis, the plastid is the site of numerous important biochemical pathways, including the synthesis of fatty acids, lipids, hormones, nucleotides, vitamins, and other secondary metabolites (Leister, 2003). In plant green tissues, plastids with a high concentration of chlorophyll and active in photosynthesis are termed chloroplasts. Plastids that are specialized for the storage and sequestration of
carotenoids are called *chromoplasts*. Non-pigmented plastids, termed *leucoplasts*, are specialized for the storage of starch, lipids and protein. The most important class of leucoplasts are *amyloplasts*, which store starch. Plastids from leaves of dark-grown plants are termed *etioplasts*. Finally, all plastids are derived from *proplastids*, which are present in meristem cells. Regardless of their developmental status, all plastids retain the potential to develop into the other plastid types (Vothknecht and Westhoff, 2001; Taiz and Zeiger, 2002).

The plastid is believed to originate evolutionarily from a single cyanobacterial endosymbiont, and during the subsequent process of symbiogenesis, most of the symbiont genes were lost or transferred to the host genome (Cavalier-Smith, 2002). In present-day land plants, the plastid genome is conserved and encodes ~100 proteins. However, this is only a very small fraction of the total number of proteins required for plastid biogenesis and function. Analysis of the Arabidopsis plastid proteome predicts the presence of approximately 1900 to 2500 nucleus-encoded gene products (Abdallah et al, 2000; Martin and Hermann, 1998). To ensure normal plastid function, plant cells have developed regulatory mechanisms to coordinate gene expression in the nuclear genome and the plastid genome (Goldschmit-Clemont, 1998; Leon et al., 1998; Rodermel, 2001b).

**Variegation mutants**

Plant variegation mutants develop sectors with different colors (green, yellow or white) in their vegetative organs (Kirk and Tilney-Basset, 1967). Cells in the green sectors of these mutants contain normal chloroplasts, while the yellow or white sectors contain cells with abnormal plastids. Studies of the pattern of inheritance of the variegation phenotype of morning glory plants led to the discovery of *non-Mendelian inheritance* in the early 1900’s,
and thus variegations have played a very important role in the history of genetics (reviewed in Granick, 1955; Kirk and Tilney-Basset, 1967).

Variegation mutants can arise by several different mechanisms (Tilney-Bassett, 1975; Kirk and Tilney-Bassett, 1978; Rodermel, 2001a; Sakamoto, 2003). Here, I classify variegation mutants into three major groups according to their molecular mechanisms, and each group will be discussed respectively in the following sections. The first class comprises variegated mutants that are caused by mutations in the plastid or mitochondrial genomes. The second mechanism involves transposable element (TE) activity. In the third mechanism, nuclear gene mutations cause the production of defective plastids is some, but not all, cells of the organism. Because plant variegations can be caused by mutations in the mitochondrial, plastid, and/or nuclear genomes, they provide excellent systems to understand the molecular basis of nuclear-organelle interactions (Bogorad et al., 1991).

**Variegations due to mutations in organelle genomes**

A typical characteristic of organelle-induced variegation mutants is maternal inheritance (non-Mendelian inheritance). The reason for maternal-inheritance is that plastids and mitochondria are usually transmitted through the female (egg), not through the male, in most higher plants. The majority of variegation mutants described by Kirk and Tilney-Basset (1967) are thought to be induced by mutations in plastid or mitochondrial genomes.

The plastome mutator (pm) variegation mutant of *Oenothera hookeri* was one of the first reported examples of an organelle DNA-induced variegation. Defective plastids in this mutant are transmitted in a maternal manner, and this trait was found to be linked to the chloroplast genome. It was proposed that *PM* might encode a protein that affects the helicity
or rigidity of the plastome (plastid genome), such as a topoisomerase or helicase. According to this hypothesis, defective PM gene products lead to template slippage during replication, resulting in deletions and duplications of the plastid genome that give rise to aberrant gene products and defective plastids (Johnson et al., 1991; Stoike and Sears, 1998). PM has not yet been cloned and molecularly-characterized.

Another well-known organelle-induced variegation is the maize nonchromosomal stripe (ncs). ncs actually refers to a group of maize variegation mutants with yellow stripes on the leaves. These mutants show typical maternal inheritance and are caused by mutations in the mitochondrial genome (Newton and Coe, 1986). To date, three different ncs mutants have been characterized at the molecular level. In ncs2, a mutation was found in the mitochondrial NAD4-NAD7 gene which encodes the NAD4 subunit of the mitochondrial electron transport chain complex I (Marienfeld and Newton, 1994). ncs3 and ncs4 are allelic and possess mutations in the gene for the mitochondrial ribosomal protein RPS3 (Hunt and Newton, 1991). In two allelic mutants, ncs5 and ncs6, the mitochondrial cytochrome oxidase subunit gene (COX2) is partially deleted (Newton et al., 1990; Lauer et al., 1990). How defective mitochondria affect chloroplast development is not well understood, but it is perhaps due to the fact that mitochondria provide energy and biosynthetic precursors to chloroplasts. For example, there is evidence that mitochondrial ATP is required for photosynthesis in the dark, and inhibition of oxidative phosphorylation of mitochondria produces photosynthetic oxygen evolution (Mackenzie and McIntosh, 1999).

Mutations in the plastid or mitochondrial genome can arise spontaneously in the organelle genome (as above) or they can be caused by mutant nuclear genes. The nuclear gene mutations are Mendelian-inherited, but the defective plastids are maternally inherited.
For instance, the Arabidopsis *chloroplast mutator (chm)* variegation mutant is caused by a nuclear gene mutation that results in a rearrangement of the mitochondrial genome (Martinez-Zapater et al., 1992). The *CHM* gene encodes a mitochondrial homolog of MutS, which is a component of the *E. coli* DNA repair system (Abdelnoor et al., 2003). It is thought that variegation arises in *chm* because alterations in the mitochondrial DNA replication and recombination system allow the accumulation of high levels of abnormal, rearranged mitochondrial genomes in some mitochondria, but not others. This results in the generation of defective mitochondria and, secondarily, defective plastids. Sorting-out of mutant mitochondrial DNAs gives rise to clones of cells containing 100% defective mitochondria (and hence all-white plastids) and to clones of cells containing genotypically wild type organelle genomes (green sectors). *chm* plants are thus genotypically chimaeric for the mitochondrial genome, but uniformly mutant for the nuclear *chm* gene.

**Variegation induced by transposable elements**

In this mechanism of variegation, transposable element (TE) insertion interrupts a nuclear gene required for normal chloroplast biogenesis, while TE excision recovers the wild type phenotype. An example of this sort of mutant is the tomato *dcl (defective chloroplast and leaf-mutable)* variegation mutant, which is caused by the *Ds* transposon (Keddie et al., 1996). In the presence of the *Ac* element, *Ds* can be excised from the insertion site in *DCL*. In cells in which *Ds* is excised, revertant green sectors form, whereas the continued presence of *Ds* in the gene gives rise to white plastids, cells and sectors. The *DCL* gene has been cloned and it encodes a chloroplast protein without known function. However, DCL might play a role in rRNA processing or ribosome assembly, since 4.5S rRNA is not processed
correctly in the white tissues of the mutant (Bellaoui et al., 2003). According to this hypothesis, failure to process rRNA results in a defect in chloroplast protein synthesis, and this leads to an early arrest of chloroplast development, thus producing white plastids and cells. An Arabidopsis ortholog of DCL has been discovered (AtDCL). Like DCL, AtDCL appears to be involved in chloroplast 4.5S rRNA maturation (Bellaoui and Gruissem, 2004).

**Variegation caused by nuclear gene mutations that do not impair organelle genomes**

The variegation phenotype of the mutants described above can be explained by the green and white sectors having different organelle genotypes. However, some nuclear gene mutations cause green and white sector formation without affecting the genotype of the organelle genome; these variegations have a uniform, Mendelian-inherited (mutant) genotype. In other words, the green and white sectors of these mutants have the same genotype but different phenotypes.

There are two types of mutants in this category according to the mode of inheritance of the plastid defect. The first group of mutants possesses permanently-defective plastids or mitochondria induced by the nuclear gene. The maize *iojap* mutant is a classic example of this type of variegation. *iojap* is a green/white striping mutant of maize in which permanently-defective, maternally-inherited plastids are produced by the action of a nuclear recessive gene. The *IOJAP* gene has been cloned and encodes a chloroplast protein with 229 amino acids that appears to be associated with the chloroplast 50S ribosomal subunit (Han et al., 1992). Consistent with this idea, *iojap* white sectors lack plastid 70S ribosomes. It is hypothesized that *iojap* plastids are permanently defective because without 70S ribosomes plastids cannot translate chloroplast DNA-encoded proteins essential for their activity. These
include the complement of ribosomal (50S and 30S) proteins that are encoded by the chloroplast genome. Hence, without ribosomes, defective plastids can not recover to a normal condition even after they are returned to a wild type nuclear genome background.

In the second group of variegation mutants, the plastids and mitochondria are not permanently damaged by the nuclear mutation. In the case of mutants with recessive mutations, when these plants are crossed with wild type plants, 100% normal-appearing F1 offspring are generated, regardless of whether the wild type or mutant is the female parent. This shows that the plastid defect is not maternally-inherited. Also, self-crossing of the (heterozygous) F1 results in a 3:1 ratio (wild type: mutant phenotype) in the F2 plants, indicating that the lesion is caused by a nuclear recessive gene. An example of this type of mutant is the Arabidopsis var2 mutant (Chen et al., 1999). var2 displays green and white sectors in its leaves, and the white sectors contain abnormal plastids with unorganized lamellar structures. The VAR2 gene encodes a chloroplast FtsH protease (Chen et al., 2000). It is hypothesized that VAR2 functions in early thylakoid membrane biogenesis. Arabidopsis immutans is another example of this type of variegation mutant. It is the focus of this dissertation and described below in detail.

It might be pointed out that because female variegated plants are able to produce totally-green F1 offspring when mated with wild type plants, defective plastids are somehow "cured", perhaps during sporogenesis (Rodermel, 2001a). How this might occur is as follows. All plastids, including defective ones (as in var2), dedifferentiate to proplastids in embryonic stem cells (Whatley, 1978). These proplastids subsequently give rise to the variety of plastid types seen in the mature plant. Thus, it is very likely that mutations like var2 do not affect the
normal life of proplastids in meristematic cells, but do affect the development process from proplastids to chloroplasts during cell differentiation.

**General features of immutans**

The Arabidopsis *im* mutant was first described and partially characterized nearly 40 years ago by Redei and his coworkers (Chung and Redei, 1974; Redei, 1963; Redei, 1967). The leaves of *im* are variegated with green sectors containing normal-looking chloroplasts and white sectors containing cells with abnormal plastids. Very interestingly, some cells in the white sectors are heteroplastidic for abnormal plastids and a few normal chloroplasts. The abnormal plastids are vacuolated and lack organized lamellar structures (Wetzel et al., 1994; Aluru et al., 2001). Also, the phenotype of *im* is light- and temperature-sensitive (Wetzel et al., 1994; Wu et al., 1999): the plants have more and/or larger white sectors under elevated temperature and light intensities. On the other hand, low light fosters green sector formation, and under very low light, mutant plants are totally green.

Morphologically, the green leaf tissues of *im* plants are thicker than those in wild type plants (Aluru et al., 2001). This is probably due to an elongation of cells in the palisade layer. By contrast, the white leaf tissues are thinner than those in wild type leaves. This is probably due to a failure of cells to expand in the palisade layer. Accompanying the "thicker leaf" phenotype, the green sectors of *im* have higher photosynthetic electron transport rates and enhanced non-photochemical quenching (NPQ) capacity compared to normal green leaves. Based on these observations, it was suggested that green sectors of *im* develop morphological and biochemical adaptations to provide nutrition to the white sectors and to protect against photodamage (Baerr et al., 2005).
Heterozygous im/IM F1 offspring resemble wild type plants, no matter whether im or wild type is used as the female parent. This indicates that defective plastids in im are not maternally-inherited and, hence, that they are not permanently-defective. Consistent with this idea, progeny from the self-crossing of im/im plants recapitulate the variegation of the parent, regardless of whether the seeds are from all-green or all-white inflorescences. Seeds collected from even extremely chlorotic siliques give rise to green, variegated and white plants (depending on ambient light intensities); completely-green siliques from im/im plants produce the same phenotypic distribution (Wetzel et al., 1994). As mentioned above, this suggests that im does not affect proplastid function in embryos, but rather, that its function is limited to differentiated plastid types.

Biochemical analysis has demonstrated that carotenoid biosynthesis is impaired in im (Wetzel et al., 1994). In these studies it was found that phytoene, a colorless C_{40} carotenoid intermediate, accumulates in the im white sectors. This suggests that the mutant plants are impaired in the activity of phytoene desaturase (PDS), the plastid enzyme that converts phytoene to zeta-carotene (Bartley et al., 1991; Cunningham and Gantt, 1998). Early experiments showed that im is not the PDS structural gene, since PDS and IM were found to be located on different chromosomes (Wetzel et al., 1994). In addition, it was found that the im gene does not affect PDS expression at the level of mRNA or protein accumulation (Wetzel and Rodermel, 1998). This suggested that IM influences the PDS step in a more indirect manner.

Carotenoid function and biosynthesis
Carotenoids belong to the terpenoid family and are broadly present in plants, algae, bacteria and some fungi (Sandmann, 2001a; Sandmann, 2001b). Plant carotenoids are red, orange, and yellow hydrophobic pigments embedded in plastid membranes. In non-photosynthetic tissues, carotenoids contribute to the color of old leaves, flowers and fruits. However, in photosynthetic organs and tissues, carotenoid color is masked by the presence of large amounts of chlorophyll (Batley and Scolink, 1995; Franka and Cogdell, 1996).

In flowers and fruits, coloration produced by carotenoids is thought to attract insects and animals to help pollination and dispersal of seeds (Batley and Scolink, 1995). In chloroplasts, carotenoids play vital and indispensable roles in photosynthesis. First, carotenoids play an important role in the light reactions of photosynthesis as accessory pigments and as components of the reaction centers (Fromme et al., 2001; Rhee, 2001). Carotenoids absorb blue light in the spectrum of 400 to 600nm, and the absorbed energy is transferred to chlorophyll. Secondly, carotenoids stabilize the lipid phase of thylakoid membranes. The interactions of carotenoid molecules and membrane lipids leads to a decrease in membrane fluidity, an increase in membrane thermostability and a lowered susceptibility to lipid peroxidation (Havaux, 1998). Thirdly, and most importantly, carotenoids play an essential role in photoprotection. When the light level exceeds the maximum that can be used by the photosynthetic apparatus (e.g. plants growing in full sunlight), singlet excited state chlorophyll ($^1\text{Chl}^*$) accumulates and is converted in triplet chlorophyll ($^3\text{Chl}^*$). $^3\text{Chl}^*$ interacts with oxygen and leads to the generation of reactive oxygen species (ROS) and photooxidation of the contents of the plastid (Demmig-Adams et al., 1996). Carotenoids protect the chloroplast against photooxidation in three ways. The first way is to quench triplet chlorophyll ($^3\text{Chl}^*$) by triplet-triplet energy transfer; this prevents the
formation of singlet oxygen (Yong and Frank, 1996). The second way is to quench singlet oxygen that has been produced, for example, by non-quenched triplet chlorophyll (Telfer et al., 1994; De et al., 1993; Tracewell et al., 2001). The third way is to dissipate excess energy of $^1$Chl* by the xanthophyll pigments. This mechanism is controlled by a process called the xanthophyll cycle (Eskling et al., 1997; Bugos et al., 1998). In this cycle, the absorption of excess light causes a high $\Delta$pH across the thylakoid membrane (low pH on the lumenal side due to proton pumping, and a high pH on the stromal side). The resulting decrease in luminal pH activates violaxanthin de-epoxidase (VDE), which converts violaxanthin to zeaxanthin (via antheraxanthin); zeaxanthin accumulates rapidly upon activation of VDE. It is thought that the VDE activation process involves it becoming tightly bound to the luminal surface of the thylakoid such that it can interact with its substrate, violaxanthin (Bugos and Yamamoto, 1996). Zeaxanthin epoxidase (ZEP) catalyzes the reverse zeaxanthin to violaxanthin conversion via a light-independent epoxidation reaction. ZEP is located on the stromal side of the thylakoid membrane and is constitutively active (Bugos et al., 1998). Thus, the level of zeaxanthin is determined by a combination of VDE and ZEP activities. Although the precise mechanism is unknown, zeaxanthin and antheraxanthin, as well as the low pH and a PSII subunit, PsbS, work together to dissipate excess energy produced by high levels of $^1$Chl* (Li et al., 2000). In summary, plants suffer severe photooxidative damage in the absence of colored carotenoids. Usually this results in an albino phenotype and the death of the organism in restrictive, high light conditions (Havaux, 1998; Tracewell 2001).

The molecular biology of carotenoid biosynthesis is well-documented and has been summarized in several reviews (Bartley and Scolnik, 1994; Cunningham and Gantt., 1998; Sandmann 2001a and 2001b; Hirschberg 2001). Carotenoids are derived from the central
isoprenoid biosynthesis pathway. The central metabolite in this pathway is isopentenyl pyrophosphate (IPP), a 5-carbon compound. Three molecules of IPP and one molecule of its isomer, dimethylallyl pyrophosphate (DMAPP), condense into one molecule of the C\textsubscript{20} geranyl pyrophosphate (GGPP). Phytoene, the colorless C\textsubscript{40} carotenoid precursor, is formed by a head-to-head condensation of two molecules of GGPP in the presence of phytoene synthase (PYS) activity. Phytoene synthesis is the first committed step of carotenoid biosynthesis. Next, phytoene undergoes two consecutive desaturation (dehydrogenation) reactions to form \(\zeta\)-carotene via phytofluene. \(\zeta\)-carotene, in turn, is desaturated in two steps to lycopene (red) via neurosporene. Two related enzymes mediate the four sequential desaturations in plants: phytoene desaturase (PDS), which is in charge of the transformation of phytoene to \(\zeta\)-carotene, and \(\zeta\)-carotene desaturase (ZDS), which is responsible for the latter two steps (\(\zeta\)-carotene to lycopene). Lycopene is the branch point in the carotenoid biosynthesis pathway, and it is cyclized to give rise to a compound with two \(\alpha\) rings (\(\alpha\)-carotene) or to a compound with one \(\alpha\) ring and one \(\beta\) ring (\(\beta\)-carotene). Both of the carotenes are yellow-orange in color. \(\alpha\)-carotene is further converted to lutein, whereas \(\beta\)-carotene is hydroxylated to the xanthophylls.

In the past few years, genes and cDNAs encoding nearly all the enzymes required for carotenoid biosynthesis have been identified and sequenced, and their products have been partially characterized (Hirschberg, 2001; Cunningham and Gantt, 1998; Schmidt-Dannert et al., 2001). However, most of the enzymes involved in the carotenoid biosynthetic pathway are membrane-associated or integrated into the membrane. They are also present in very low abundance. These features along with their sensitivity to detergents (which must be used for
solubilization) make the isolation and purification of active enzymes very difficult and thus hinder the elucidation of their structure and function. One of the goals of this thesis is to study the structure and function of IM, also a membrane-bound protein (see later).

Phytoene desaturase

Little is known about the regulation of the carotenoid pathway. There is evidence that phytoene desaturation is the rate-limiting step in cyanobacterial carotenoid biosynthesis (Chamovitz et al., 1993). However, the PYS step appears to be the rate-limiting step in plants (Hirschberg, 2001). I will briefly discuss phytoene desaturase (PDS) because of its relevance to the mechanism of im variegation and IM function.

Genes for PDS have been cloned from various higher plants, including Arabidopsis (Scolnik and Bartley, 1993), tomato (Pecker et al., 1992), soybean (Bartley et al., 1991), daffodil (Al-Babili et al., 1996), and citrus (Kita et al., 2001). PDS enzymes are well-conserved in amino acid sequence among different species. For example, PDS from Synechococcus PCC7942 and Arabidopsis share 65% amino acid identity and 79% similarity. Arabidopsis PDS encodes a polypeptide of 566 amino acids (63 kD) with the N-terminal 92 amino acid sequence serving as a chloroplast transit peptide (Scolnik and Bartley, 1993).

Although the predicted amino acid sequences of PDS are not particularly hydrophobic, PDS is associated with the thylakoid membrane (Bramley, 1985). However, a soluble form of phytoene desaturase is found prior to membrane binding, but it is not active (Al-Babili et al., 1996; Bonk et al., 1997). From detergent titration experiments, PDS was found to be a peripheral membrane protein rather than an integral membrane protein (Schledz et al., 1996). Although there is evidence suggesting that PDS might be associated with chloroplast
envelope membranes (Lutke-Brinkhaus et al., 1982), results from immunogold localization experiments demonstrated that PDS is mainly associated with thylakoid membranes (Linden et al., 1993). FAD incorporates into PDS before or at the time of membrane integration, and the membrane-associated enzyme is not active without FAD. Enzyme assays showed that PDS activity does not require additional FAD, suggesting that tightly bound FAD is a cofactor (Schledz et al., 1996).

It was early thought that O$_2$ is an electron acceptor of the PDS reaction, but that it is not a direct acceptor (Cunningham and Ganyt, 1998; Nievelstein et al, 1995). Mayer et al (1990) reported that in an anaerobic environment, oxidized quinine is required for PDS activity, whereas the reduced form is ineffective. It was later found that the Arabidopsis $pds1$ and $pds2$ mutants display an albino phenotype and accumulate phytoene due to a blockage in the plastoquinone (PQ) biosynthetic pathway. This finding strongly suggested that PQ is an essential component required for phytoene desaturase activity (Norris et al., 1995; Norris et al., 1998). This is consistent with the biochemical demonstration that PQ can serve as a direct electron acceptor for higher plant PDS, while NADH is also an effective electron acceptor for cyanobacterial PDS. The well-known PDS inhibitor, norflurazon, inhibits PDS by replacing PQ through a competition mechanism (Breitenbach et al., 2001).

In summary, prior to the cloning of $IM$ in 1999 (Wu et al., 1999), the working hypothesis was that electrons from phytoene are transferred to the PQ pool via PDS, and then from PQ to O$_2$ in one or more, uncharacterized redox reactions.
IM and IM homologs

The IM gene was cloned by map-based methods and also by transposon-tagging (Wu et al., 1999; Carol et al., 1999). The IM gene is 2557 bp long and contains 8 introns and 9 exons. The IM cDNA is a 1.4 kb fragment and consists of a 162 bp 5’UTR, a 1053 bp open reading frame and a 232 bp downstream 3’UTR sequence. The IM cDNA encodes a protein of 347 amino acids with a calculated molecular weight of 40.5 kD. It is predicted that the first 50 amino acids comprise an N-terminal transit sequence that targets the protein to the chloroplast. Chloroplast import assays directly demonstrated that the IM precursor can be imported into the chloroplast (Carol et al., 1999; Fu et al., 2005). After cleaving the chloroplast transit peptide, the mature IM protein is a 35 kD membrane protein. Further localization analyses revealed that IM is located in the thylakoid membranes of stromal lamellae, but not in the granal lamellae, and that the protein faces toward the stromal side of the thylakoid membrane (Joet et al., 2002; Lennon et al., 2003).

The tomato homolog of IM, the GHOST gene, has also been cloned in our lab, as well as in another lab (Barr et al., 2004; Josse et al., 2000). The GHOST genomic DNA is about 5 kb long and the gene contains 8 introns and 9 exons, identical to the number and location in the IM genomic sequence. The big difference between GH and IM is in the intron sizes. The GHOST protein contains 366 amino acids with a predicted molecular weight of 42.1 kD. It bears 67% amino acid sequence identity to IM, with most of the variability in the putative N-terminal plastid targeting sequence. IM homolog cDNAs have also been isolated in pepper (Josse et al., 2000) and rice (Kong et al., 2003). Genomic Southern blotting analyses and database searches have demonstrated that IM, GHOST, and the rice OsIM1 gene (the rice IM homolog) are single-copy genes (Wu et al., 1999; Carol et al., 1999; Barr et al., 2004; Kong
et al., 2003). IM homologs are also found in algae (Archibald et al., 2003) and some cyanobacteria (Kaneko et al., 2001; Nakamura et al., 2003). The IM protein and its homologs are also called PTOX, because they display plastid terminal oxidase activity (Josse et al., 2000; Carol and Kuntz, 2001) (see below).

Database searches revealed that the IM protein bears similarity to alternative oxidase (AOX). Alternative oxidases are mitochondrial inner membrane proteins found in higher plants, as well as in some algae, fungi, and protists (Siedow and Umbach 1995, Vanlerbergh and McIntosh 1997). The recent finding of an AOX homolog in an α proteobacterium, which is thought to be the ancestor of mitochondria (Gray et al., 1999), as well as the recent finding of a PTOX homolog in a cyanobacterium, which is thought to be the ancestor of plastids, give insight into the once mysterious question: what are the origins of AOX and PTOX (Atteia et al., 2004; Stenmark and Nordlund 2003)? Arabidopsis AOX and PTOX share ~26% amino acid identity, while α-proteobacterial AOX shares about 50% amino acid identity with eucaryotic AOX, and cyanobacterial PTOX shares a similar (50%) amino acid identity with eucaryotic PTOX (Finnegan et al., 2003; McDonald et al., 2003). Detailed phylogenetic analyses have revealed that a common progenitor of AOX and PTOX arose in an ancestral prokaryote, and then diverged in cyanobacterial and α-proteobacterial lineages. These further evolved into mitochondrial AOX and plastid PTOX as a result of endosymbiotic events (Finnegan et al., 2003).

**AOX and PTOX structure**

Unlike animal mitochondria, plant mitochondria have a bifurcated electron transfer chain. In addition to the cytochrome respiratory pathway found in all eukaryotes, plants have
a second, alternative pathway that diverges from the main respiratory electron transfer chain at the ubiquinone site (Vanlerberghe and McIntosh, 1997). The terminal enzyme in the alternative pathway is called alternative oxidase (AOX). It is present in the inner mitochondrial membrane. The substrates of AOX are ubiquinol and O₂, and iron is essential for activity. Because AOX is a membrane protein, it is hard to generate a good crystal form for X-ray analysis.

Until recently, little was known about the overall structure of the AOX active site. Siedow et al (1995) proposed a structural model of AOX based on the “RNR R2” class of di-iron carboxylate proteins (named after the R2 subunit of ribonucleotide reductase). The active sites of RNR R2-type proteins consist of a binuclear iron center coordinated by two histidines and four carboxylate residues. Their model suggested that the AOX protein is an integral membrane protein containing two transmembrane domains, with the N- and C-termini exposed to the matrix side of the membrane.

Andersson and Nordlund (1999) and Berthold et al (2000) pointed out that the Siedow model did not agree with the structure of other well-characterized di-iron proteins, and based upon a much larger data set of AOX sequences than was available to Siedow et al, they proposed a revised structural model of AOX (the “ANB” model). In the ANB model, the hydrophobic regions of AOX are not transmembrane segments but, rather, AOX is proposed to be an interfacial membrane protein with an active site contained within a four helix bundle. EPR spectroscopy data proved that there is a coupled di-iron center present in this site (Berthold et al., 2002), and in vitro mutagenesis analysis of the proposed Fe-ligands of AOX using prokaryotic model systems support this model (Bertold et al., 2002; Albury et al., 1998; Albury et al., 2002; Ajayi et al., 2002; Chandhuri et al., 1998; Affourtit et al., 2002).
Taking advantage of an *in vitro* assay of IM activity and the availability of null *im* alleles, I used the IM protein as model system to test the functional significance of the putative Fe-ligands of AOX ad PTOX *in vitro* and *in planta*. These experiments showed that the six Fe-binding sites do not tolerate change, even conservative ones (Fu et al., 2005) (see Chapter 2 of thesis). I also found that a 16 amino acid fragment, which corresponds to exon-8 of the genomic sequence and that is not present in AOX, is important for IM activity and stability.

**The role of AOX in oxidative stress**

The physiological role of alternative oxidases in plant non-thermogenic tissues has been an enigma and is still not fully understood. It is well known that AOX protein levels can be induced by several treatments such as wounding, chilling, drought, osmotic stress and pathogen attack. AOX can also be induced by treatment with salicylic acid, H$_2$O$_2$ or inhibitors of the respiratory chain. In general, any condition that inhibits or decreases the activity of the main respiratory chain induces the alternative pathway (Vanlerberghe and McIntosh, 1997). Such observations have been interpreted by the hypothesis of “overflow”. By serving in an overflow capacity, TCA cycle activity can occur under conditions when the cytochrome pathway is restricted (Lambers, 1982; Moore and Sideow, 1992). Recently, a new idea about AOX function was proposed, in which AOX allows flexible control of ATP synthesis to maintain growth rate homeostasis (Hansen et al., 2002; Moore et al., 2002).

It also has been proposed that plant AOXs play a role in plant defense against oxidative stress (Purvis 1997; Wagner and Moore 1997; Maxwell et al., 1999; Millar, 2001). This proposal was based upon the observation that all conditions resulting in an increase of oxidative stress also induce AOX activity. In other words, AOX can function as a mechanism
to decrease the formation of reactive oxygen species (ROS), the unavoidable byproducts of aerobic metabolism (Moller, 2001). Because of the highly reactive and toxic properties of ROS, plant cells have to maintain a dynamic balance between the production of ROS and the scavenging of ROS, even under normal physiological conditions. Under stress environments, a decrease of scavenging and/or an increase in the production of ROS will lead to an accumulation of ROS, which will cause oxidative damage to cells (Bowler et al., 1992; Apel and Hirt, 2004). Plant mitochondria and chloroplasts are the two organelles most active in oxygen metabolism, so they are the two major sources of ROS. Experimental evidence that AOX is able to lower mitochondrial ROS production (Purvis, 1997; Maxwell et al., 1999) and that deletion of AOX increases plant susceptibility to ROS (Robson and Vanlerberghe, 2002) generally support this idea. So, too, do experiments in which overexpression of AOX in Arabidopsis leads to the generation of plants that are more tolerant of ROS and that show a higher resistance to low temperature (Umbach et al., 2005; Fiorani et al., 2005). AOX is thought to decrease ROS production in two ways: by preventing the reduction of O$_2$ to O$_2^-$, and by reducing the overall level of O$_2$ (Mittler, 2002).

**IM and chlororespiration**

Recent experiments have supported the idea that IM serves a global role in plastid metabolism besides its involvement in carotenoid biosynthesis (Aluru et al., 2001; Baerr et al., 2005). For example, IM has been suggested to be a component in the arsenal of plastid responses to oxidative stress, likely as a “safety valve” for the dissipation of excessive electron flow (Niyogi, 2000; Rizhsky et al., 2002). In addition, IM appears to be the terminal
oxidase of the so-called “chlororespiration” process, an alternative electron transfer chain on the thylakoid membrane (Josse et al, 2000; Carol and Kuntz 2001).

Chlororespiration refers to a respiratory electron transfer chain coexisting with the photosynthetic electron transfer chain on chloroplasts thylakoid membranes that involves the non-photochemical reduction of plastoquinone (PQ) by a chloroplast NADH dehydrogenase, and the oxidation of plastoquinol (PQH₂) by a terminal oxidase (Bennoun, 1982; Bennoun, 2002). This chloroplast respiratory activity was suggested to be a relic from the chloroplast ancestor during evolution (Scherer, 1990). The chloroplast ancestor, cyanobacteria, has both a photosynthetic electron chain and a respiratory electron chain coexisting on the thylakoid membrane; two electron transport chains share some intermediates such as PQ and cytochrome b₆/f (Cooley et al., 2000). Evidence for such a non-photochemical reduction of the PQ pool is available in *Chlamydomonas* and higher plants (Bennoun, 1983; Bennoun, 1994; Garab et al., 1989; Lajko et al., 1997).

Identification of the molecular components of chlororespiration has been difficult. The discovery of *ndh* genes in the chloroplast genome with similarities to the mitochondrial NADH:ubiquinone oxidoreductase suggested that a chloroplast NADH dehydrogenase (NDH) complex might function in chlororespiration (Burrow et al., 1998; Kofer et al., 1998; Casano et al., 2000). Recent biochemical and genetic analyses of *ndh* mutants have provided strong evidence for Ndh complex function in the reduction of PQ (Guedeney et al., 1996; Horvath et al., 2000). Further, the similarity of IM to mitochondrial AOX and its function as a PQH₂ oxidase suggested that IM might be involved in the oxidation of PQH₂ and that it could be the elusive plastid terminal oxidase of chlororespiration. Evidence for IM as the plastid terminal oxidase comes from experiments in *Chlamydomonas* and tobacco (Cournac
et al., 2000a and 2000b; Joet et al., 2002; Nixon 2000). Using Chlamydomonas mutants deficient in PSI and the cyt$b_6/f$ complex, it was shown that PQH$_2$ oxidation in the dark is due to a plastid quinol oxidase that is sensitive to propyl gallate; AOX and IM are sensitive to propyl gallate. More conclusive evidence for a role of IM in chlororespiration comes from transgenic tobacco plants that overexpress the IM gene. In these experiments, IM was found to be active in tobacco chloroplasts and was shown to oxidize PQH$_2$ using molecular oxygen as a terminal acceptor in the dark. Recent findings with IM fit the chlororespiration hypothesis: IM has PQH$_2$ oxidase activity \textit{in vitro} and \textit{in vivo} (Cournac et al., 2000a and 2000b; Josse et al., 2002).

Under normal physiological conditions, chlororespiration appears as a quantitatively minor process compared to the activity of photosynthesis in higher plant chloroplasts. It has been estimated that chlororespiratory electron flow represents approximately 2% of the maximal photosynthetic electron flow (Peltier and Cournac, 2002). What are the physiological roles of the chlororespiration pathway in the plastid? Three possible roles are thought to be played by chlororespiration (Nixon, 2000; Peltier and Cournac, 2002). First it may balance the reduction potential of the plastoquinone pool, and reduce the possibility of generation of ROS. In other words, chlororespiration may be one of the safety valves for photosynthesis when chloroplasts absorb excess light energy (Niyogi, 2000). Secondly, chlororespiration may play an important role in gene expression and chloroplast biogenesis, since chlororespiration can regulate the reduced/oxidized state of the PQ pool. The reduced/oxidized state of the PQ pool plays a role in the regulation of gene expression (Pfannschmidt et al., 1999). Thirdly, chlororespiration may play a role in energy metabolism and ATP synthesis when the photosynthetic electron transfer chain is not functional. For
example, in the early stages of chloroplast biogenesis, in the non-photosynthetic plastid, or under dark conditions, the contribution to energy metabolism of chlororespiration may become important (Peltier and Cournac 2002).

Regardless of whether IM is involved in the process of chlororespiration or not, there is no doubt that IM is a plastoquinol oxidase. Like the mitochondrial electron transfer chain, in which the ubiquinone pool has multiple electron donors and two electron acceptors (Moller, 2001; Rasmusson et al., 2004), the PQ pool in photosynthetic electron transfer chain may have multiple electron donors and two acceptors as well. The electron donors of the PQ pool include PSII, PDS, and probably NADH dehydrogenase; and the electron acceptors are PTOX and PSI.

**Variegation mechanism of immutans**

A model to interpret the *immutans* variegation mechanism has been proposed based on IM gene function and on the biochemical and genetic behavior of *im* plants (Wu et al., 1999). During the very early stages of chloroplast development, thylakoid membranes and components of the photosynthetic apparatus start to be synthesized (Vothnecht and Westhoff, 2001). IM’s role in the phytoene desaturation process is crucial for plastid development: IM functions as an oxidase to convert PQH$_2$ to PQ, which serves as the electron acceptor of PDS activity. In the absence of IM, PDS activity is not functional, and carotenoid biosynthesis is blocked, resulting in photooxidation of the plastid and the formation of white sectors. At a later stage of plastid differentiation, once chloroplasts are formed and the photosynthetic electron chain is functional, IM does not seem to be important, because a functional photosynthetic electron chain is able to replace IM’s role in PDS activity. The green sectors
of variegated plants could originate from cells that have escaped irreversible photooxidative
damage by some unknown mechanism. This model suggests that in im mutants, there is a
threshold of electron transport activity compatible with the formation of green sectors: below
this threshold green sectors form, but above this activity the PQ pool becomes reduced,
phytoene accumulates, and unquenched ROS are produced. Consequently, the outcome of
development in IM-deficient plastids is either a white, photooxidized state or a functional,
green state.

This model explains the role of IM in phytoene desaturation very well. And it also
addresses very well why IM is dispensable after the formation of thylakoids. However, it is
still a mystery how a leaf can develop two distinct tissues under a uniform genetic
background. In this thesis, I applied molecular genetics tools to explore the molecular
features of IM structure and its functional roles. A hypothesis was proposed to interpret the
variegation mechanism in im plants based on my understanding of the IM protein and im
plants.

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CHAPTER 2. SEQUENCES REQUIRED FOR THE ACTIVITY OF PTOX (IMMUTANS), A PLASTID TERMINAL OXIDASE: IN VITRO AND IN PLANTA

MUTAGENESIS OF FE-BINDING SITES AND A CONSERVED SEQUENCE THAT CORRESPONDS TO EXON 8

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ABSTRACT

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 plastoquinone pool. PTOX and AOX are diiron carboxylate 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 of null immutans alleles for the latter studies. These experiments showed that the six Fe-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.

INTRODUCTION

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 (ROS) 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 C_{40} intermediate in carotenoid biosynthesis (10). This indicates that im is impaired in the activity of phytoene desaturase (PDS), which converts phytoene to \( \zeta \)-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 plastoquinone (PQ) (via PDS) and then from PQ to oxygen (via IM) (1, 2). It was further suggested that a lack of IM (as in im) results in overreduction of the PQ 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; D. Rosso, A. Fu, S. Rodermel, and N. Huner, unpublished data). 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 carboxylate 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 (Figures 1A 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 Fe-ligands of AOX using prokaryotic model systems to test function (such as a heme-deficient E. coli strain) (3, 26-
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 (Figure 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/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 Fe-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 Fe-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 (ABRC, 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 lab (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 NheI sites of pET-11a (Novagen, Madison, WI), and the QuikChange™ Site-Directed Mutagenesis protocol was used to generate mutations (Stratagene, LaJolla, 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 *A. thaliana* IM sequence and the amino acid to which it was changed (e.g., H299A: his 299 was changed to ala).

The primers listed in Tables 1 and 2 were used to produce mutations in the Fe-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 planta** - 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 cDNAs were cloned behind the CaMV 35S promoter in the binary vector pBI121, 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 1× MS salts (pH 5.7), 1% sucrose and 50 μg ml⁻¹ 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 OD₆₀₀ of 0.6. Protein expression was induced by the addition of 1.6 mM IPTG (final concentration). After two hours, the cells were harvested by centrifugation (3,000g 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 PMSF and 0.3% protease inhibitor cocktail (Sigma, St.Louis, MO). The resuspension was sonicated briefly and unbroken cells were removed by centrifugation (3,000g for 10 min at 4°C). The supernatant was then centrifuged in a Beckman L8-70W ultracentrifuge (200,000g for 2 hours at 4 °C) and the membrane pellet was resuspended in a solution containing 0.75 M sucrose, 0.2 M Tris-HCl (pH 7.5). The concentration of proteins in the pellet was measured using the Bio-Rad protocol (Hercules, CA) with BSA as a standard.

Oxygen consumption was monitored with a Clark O₂ electrode (Hansatech, Norfolk, England). Assays were conducted at 25°C with 100 - 200 μg of membrane protein in a 1.5 ml reaction mixture containing 50 mM Tris-maleate (pH 7.5), 10 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 0.2 mM decyl-plastoquinone (2,3-methyl-5decyl-1,4-benzoquinone, from Sigma). The reactions were initiated by the addition of NADH (1 mM final concentration),
followed by KCN (3 mM final concentration) and n-propyl gallate (3,4,5-tri-hydroxy-benzoic acid-n-propyl ester) (0.5 mM final concentration), all available from Sigma.

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 PhosphorImage 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 2,600g for 5 min. The pellet was washed with 10 mM 3-(N-morpholino) propane-sulfonic acid (MOPS) (pH 8.0). Following centrifugation (10,000g 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 previously outlined (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 (1:3000 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 BamH1 and Nhe1 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 TNT Quick Master, 20 uCi [35S] methionine and 1 μg 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/L BSA, 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 2,600g. The resulting crude chloroplast pellet was resuspended in GR buffer, then loaded onto a Percoll gradient. After centrifuging 10 min at 10,500g, 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% BSA, 1.5 mM DTT, 1.2 mg antipain (Sigma), 5 mM ATP and 40 μl [35S]-labeled IM transcription/translation products. The import assay was initiated by adding chloroplasts (corresponding to 200 μg 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 three hours.

RESULTS AND DISCUSSION

Mutagenesis of putative Fe-binding ligands in vitro - Berthold and colleagues have proposed that the structure of PTOX is similar to that of AOX (Figure 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. Figure 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).

Figure 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 Fe-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 (Figure 1A). Taken together, these data support the Berthold/Andersson/Nordlund model of PTOX (24).

As a first approach to test the functional significance of the six putative Fe-ligands of PTOX, we conducted site-directed mutagenesis experiments using an in vitro activity assay 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 Fe-binding ligands of Arabidopsis PTOX (IM gene product) were changed to conservative or non-conservative amino acids: the glu residues were changed to ala (nonconservative) or asp (conservative), and the his residues were changed to ala (nonconservative), or asn (conservative with respect to the space occupied, but non-conservative with respect to charge). Glu was also changed to his (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 Fe 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.

Figure 2 shows the O₂ consumption traces of four representative experiments to test whether H178 (in helix 2) is essential for PTOX activity. In contrast to membranes from non-transformed E. coli (Figure 2A and Figure 3A, lane 1), membranes from cells that contain wild type PTOX display cyanide-resistant O₂ consumption (Figure 2B and Figure 3, lane 2). This activity is inhibited by n-PG, suggesting that it arose from PTOX. Figure 2D (and Figure 3A, lane 10) reveals that mutation of H178 to ala (H178A) nearly abolishes
cyanide-resistant O₂ consumption, but that mutation of the adjoining his residue to ala (H177A) has no affect on consumption (Figure 2C, Figure 3A, lane 9). n-PG sensitive O₂ consumption is also blocked by a partially-conservative substitution of H178 to asn (H178N) (Figure 3, lane 12), as well as by alteration of his to glu (H178E) (Figure 3, lane 11). Taken together, these data indicate that H178 is requiredl for PTOX activity and/or stability, and that his at this site is essential.

Results similar to those in Figure 2 were obtained for each of the other five putative Fe-ligands (Figure 3A):


2. O₂ consumption is inhibited by conservative substitutions of glu to asp (E136D, E175D, E227D, E296D) (lanes 4, 7, 14, 17).

3. The Fe-ligands do not appear to be interconvertible, i.e., O₂ consumption is inhibited when glu residues are converted to his or vice versa (E136H, E175H, E227H, E296H, H299E) (lanes 5, 8, 15, 18, 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 carboxylate residues that reside near the Fe-ligands have little, if any, affect on O₂ consumption (E298A, lane 19). This suggests that the introduced Fe-binding site mutations do not affect the conformation of the active site.
In summary, the in vitro mutagenesis studies suggest that the six putative Fe-binding sites of PTOX do not tolerate change. In fact, the only changes that do not impact activity are those that involve nearby carboxylate residues (H177A and E298A, lanes 9 and 19).

One possibility to explain the lack of PTOX activity is that the mutant proteins are not expressed in E. coli, or that they are expressed but degraded. To examine this question, we performed Western immunoblot analyses on membrane proteins from the various transformants using a polyclonal IM antibody (17). PTOX was detected in the membrane but not soluble protein fractions, indicating that the various mutations do not likely compromise the incorporation of PTOX into E. coli membranes (data not shown). Figure 3B reveals that the transformed cells accumulate the mutant proteins (lanes 3-24) at levels found in cells that express the wild type enzyme (lane 2); control transformants with the empty vector do not accumulate PTOX (lane 1). These data indicate that the mutant proteins are expressed and stable in E. coli.

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 Fe-binding ligands have wild type levels of cyanide-resistant O₂ consumption.

Mutagenesis of putative Fe-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 Figure 3A). The data from ten representative transformants (10 different constructs) are shown in Figure 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 carboxylate residues) resemble wild type, suggesting that these two mutations do not impact PTOX activity *in planta*.

Figure 3D shows that the mutants in Figure 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 Fe-binding sites are deleterious to PTOX
activity in planta. The only two mutations that do not seem to affect activity are those in carboxylate residues that reside near the Fe-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 Fe-ligands of PTOX are required for enzyme activity. In addition, these residues do not tolerate change, even conservative ones. This might explain why these residues are perfectly-conserved in all AOX and PTOX proteins examined to date.

The present findings are in agreement with previous mutagenesis studies on AOX, in which the functional significance of five of the six Fe-binding sites has been examined (the helix 2, 3 and 4 ligands) (26–30). Mutagenesis of the sixth ligand (E183 on helix 1) has not been reported. Some of the mutagenesis data are equivocal. For example, Berthold et al. (26) found that mutations in four of the Arabidopsis AOX1a Fe-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 identification of E222 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 E222 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 Fe (E222 and E273, corresponding to E175 and E227 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: \( \alpha \)-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 (cys-127 of Arabidopsis AOX1A, see Figure 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", Figure 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 1A show that PTOX proteins lack the AOX-specific regulatory regions, i.e., the regulatory cys (or ser), the "dimerization domain", and the fungal-specific N-terminal domain (Figure 1A). This confirms earlier observations (1, 2, 38). However, our comparisons further revealed that PTOX contains a conserved 16 amino acid insertion in (or near) the active site that is not found in AOX from any source. This sequence is present in all the species in Figure 1C with the exception of Synechococcus (where it is truncated) and P. mainanus (where it is totally absent). Alignments show that seven of the 16 residues are
identical in this sequence across species (Figure 4). Other amino acids are also highly
conserved (e.g., E268 is found in all species except *Chlamydomonas*). Interestingly, the 16
amino acid sequence corresponds precisely to Exon 8 of the higher plant genomic sequence
and is located between helix 3 and helix 4 (Figures 1A and 1B). Database searches revealed
that this sequence is not found in other proteins and that it does not contain or comprise a
known protein motif.

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, n-PG sensitive O₂ consumption activity (as Figures 2 and
3A). They were also introduced into *im* to test their *in planta* activity (as Figure 3C).

Figure 5A shows that deletion of all or part of Exon 8 nearly abolishes *in vitro* PTOX
activity, whereas activity is not appreciably affected by mutations in the eight conserved
amino acids. Protein expression in *E. coli* is similar for all the mutant proteins (Figure 5B).
As expected, the DEX8, D1-8 and D9-16 proteins migrate at a lower molecular weight than
the normal-sized protein.

Figure 5C shows that mutations in the eight conserved amino acids do not impact
PTOX activity *in planta* inasmuch as the T₂ plants resemble wild type. In contrast, DEX8,
D1-8 and D9-16 T₂ plants are variegated. This suggests that the deleted sequences are
essential for activity. The various transformants in Figure 5C have wild type levels of IM
protein, and the deletion mutant proteins are smaller than normal, again as anticipated (Figure 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 ABRC) and that lacks Exon 8. This allele was generated by EMS and Exon 8 is perfectly deleted from this strain (data not shown). 3639 and wild type Columbia are shown in Figure 6A. Northern blot analyses revealed that IM mRNAs are expressed at normal levels in 3639 (Figure 6B), however the protein product is undetectable by Western blot analysis (Figure 6C). The phenotype of 3639 resembles that of other alleles of im that are predicted to be null (1, 10).

In contrast to the normal accumulation of protein that occurs when the DEX8 construct is overexpressed in im (Figure 5D, above), the lack IM protein in 3639 (i.e., which is also predicted to contain an Exon 8 deletion) suggests that accumulation of the mutant protein is regulated post-translationally in 3639, but not in the overexpressor. To test this hypothesis, we examined the stability of the mutant protein after import into isolated chloroplasts. The DEX8 and wild type IM constructs were transcribed and translated in vitro using [35S] methionine, and the labeled proteins were then imported into 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 Figure 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 Figure 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 three hour chase. Figure 7A reveals that both proteins decrease in amount during the chase, and that after three hours, the levels of the wild type protein decrease about 20% versus 60% for the mutant protein (Figure 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.

ACKNOWLEDGEMENT

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Structures of AOX and PTOX.

**A.** AOX and PTOX have four helices that contain six putative Fe-ligands in the diiron center at the active site (21-23). The four conserved helices with their E and H 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
"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 EXXH motifs on helices 2 and 4 (oriented anti-parallel to each other), and two E 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 *Arabidopsis thaliana* (At IM, gi 4138855), *Capsicum annuum* (pepper Ptf, gi 9937103), *Lycopersicon esculentum* (tomato GHOST, gi 9937101), *Oryza sativa* (rice IM, gi 21105122), and *Triticum aestivum* (wheat IM, gi 9837152). The algal sequences are from *Bigelowiella natans* (gi 32307546) and *Chlamydomonas reinhardtii* (gi 201492540), and the cyanobacterial ones are from *Gloeobacter violaceus* PCC7421 (gi 35211163), *Synechococcus sp.* WH8102 (gi, 33865421), *Anabaena variabilis* sp. ATCC29413 (gi, 4550896), *Prochlorococcus marinus* MED4 (gi, 33860894) and *Anabaena variabilis* sp. PCC7120 (gi, 17229588). Full-length amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). *Arabidopsis thaliana* Aox1a (AtAOX1a, gi 3915639) served as an outgroup. Included are all PTOX homologs in the Genbank non-redundant protein database as of 7/1/05.

**Figure 2.** Oxygen consumption profiles of *E.coli* membranes that contain PTOX.

**A.** Control membranes from nontransformed cells.

**B.** Membranes containing wild type PTOX.

**C.** Membranes containing the mutant H177A PTOX.
D. Membranes containing the mutant H178A PTOX.

**Figure 3 Mutagenesis of active site Fe-binding ligands *in vitro* and *in planta***

A. The oxygen consumption rates were determined from the slopes of O₂ traces following the addition of 1mM NADH, 3mM KCN, and 0.5mM propyl gallate (PG) as shown in Figure 2. The PTOX activity is represented by the oxygen consumption rates in the presence of 3mM KCN minus the oxygen consumption rates in the presence of 0.5mM PG. The rates are the average values of 4 measurements (+/- SD). Lane 1, *E. coli* with empty pETlla vector; Lane 2, *E. coli* with the pETlla/PTOX (wild type). Lanes 3-22, *E. coli* containing the expression vector with the following mutations: Lane 3, E136A; Lane 4, E136D; Lane 5, E136H; Lane 6, E175A; Lane 7, E175D; Lane 8, E175H; Lane 9, H177A; Lane 10, H178A; Lane 11, H178E; Lane 12, E178N; Lane 13, E227A; Lane 14, E227D; Lane 15, E227H; Lane 16, E296A; Lane 17, E296D; Lane 18 E296H; Lane 19, E298A; Lane 20, H299A; Lane 21, H299E; Lane 22, H299N.

B. Total membrane proteins were isolated from transformed *E. coli*, 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 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 168 *bone fide* transformation events were examined (at least eight per construct); the figure shows
ten representative $T_2$ generation seedlings (ten different constructs). All plants were
grown for 3-4 weeks under continuous light (60-90 $\mu$mol m$^{-2}$ sec$^{-1}$).

**D.** Western immunoblot analyses were conducted using 5 $\mu$g 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 Figure 2. "—"
denotes a gap in the sequence. Degree of conservation ("consensus") correlates with
font size.

**Figure 5.** Mutagenesis of PTOX exon 8 sequences *in vitro* and *in planta*.

**A.** The figure legend is the same as for Figure 3A. The rates are the average values of
four measurements.

**B.** The figure legend is the same as for Figure 3B.

**C.** The figure legend is the same as Figure 3C.

**D.** The figure legend is the same as Figure 3D.

**Figure 6.** Wild type and *immutans* (3639 allele) plants.

**A.** Mutant strain 3639 was obtained from the ABRC and is an allele of *immutans* that
lacks Exon 8.
B. Northern blots analyses were conducted using 10 ug of total cell mRNAs from WT leaves and from green sectors of 3639. The nitrocellulose filters were probed with an IM cDNA (8).

C. Western immunoblot analyses were conducted using 5 ug 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.

Figure 7. Stability of wild type PTOX and PTOX lacking Exon 8.

A. Chloroplast import assays were conducted as described in the Materials and Methods. Equal volumes of $^{35}$S-methionine- labeled wild-type and mutant (DEX8) proteins were removed from reaction mixtures and electrophoresed through 12.5% SDS-PAGE gels. The gel was dried then exposed to X-ray film.

B. Bands in the autoradiographs corresponding to the mature protein were quantified by Quantity One software (Bio-Rad), and the relative amounts of label were calculated (100% is the “0 hour time point” for WT and DEX8). The assays were repeated four times.
Table 1 Primers for the introduction of mutations in the six putative Fe-binding sites

<table>
<thead>
<tr>
<th>mutation</th>
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<tr>
<td>E136A</td>
<td>5'-gcaaggtttttgcagctagagctg-3'</td>
</tr>
<tr>
<td>E136D</td>
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**FIGURE 4**
FIGURE 5
Figure 6

A

B

C

Figure 7

A

B

Relative Amount of Label

WT DEX8  WT DEX8  WT DEX8
CHAPTER 3. CONSERVED ACTIVE SITE SEQUENCES IN ARABIDOPSIS

PLASTID TERMINAL OXIDASE (PTOX): \textit{IN VITRO AND IN PLANTA}

MUTAGENESIS STUDIES

A paper submitted to the Journal of Biological Chemistry

Aigen Fu and Steven R. Rodermel

ABSTRACT

The plastid terminal oxidase (PTOX) is distantly-related to the mitochondrial alternative oxidase (AOX). Both are members of the diiron carboxylate quinol oxidase (DOX) class of proteins. The active sites of PTOX and AOX contain 20 highly-conserved amino acids, six of which are Fe-binding ligands. We have previously used \textit{in vitro} and \textit{in planta} activity assays to examine the functional importance of the Fe-binding sites. The \textit{in planta} procedure was based on the ability of mutant enzymes to complement the Arabidopsis \textit{immutans} mutant, which lacks PTOX. In this manuscript we conduct alanine scanning mutagenesis on the 14 other conserved sites using our \textit{in vitro} and \textit{in planta} assay procedures. We found that the 14 sites fall into three classes: i) A139, E171, N174, L179, P216, A230, D287 & R293 are dispensable for activity; ii) Y234 & D295 are essential for activity; and iii) L135, P142, H151, and Y212 are important but not essential for activity. Our data are consistent with the proposed role of some of these residues in active site conformation and/or catalysis. Titration experiments showed that downregulation of PTOX to \(-3\%\) of wild type levels did not compromise plant growth, at last under ambient growth conditions. This suggests that PTOX is normally in excess, especially early in thylakoid membrane biogenesis.
INTRODUCTION

Alternative oxidase (AOX) is a terminal oxidase that functions in the alternative pathway of mitochondrial respiration (1). It catalyzes the four-electron reduction of oxygen to water and branches from the cytochrome pathway at the level of the quinone pool (1-3). The alternative pathway is found in plants, algae, fungi and some protists, but not animals. It is thought that AOX is activated when the cytochrome pathway becomes saturated, for instance, during oxidative stress when the inner membrane is highly energized and prone to the production of reactive oxygen species (ROS) (3-7).

The IMMUTANS locus of Arabidopsis was recently cloned and identified as a plastid homolog of AOX (8, 9). IM-like proteins have subsequently been found in a diverse array of plant, algal and cyanobacterial species; like AOX, IM does not appear to be present in animals (summarized in 10). Also similar to AOX, IM functions as a terminal oxidase, transferring electrons from the plastoquinol (PQ) pool to molecular oxygen (11-14). IM is thus frequently designated “PTOX” (plastid terminal oxidase). [To avoid confusion, we will use the term PTOX in this paper, and IMMUTANS when describing the Arabidopsis gene for PTOX.] Current thinking is that PTOX is an important alternative electron sink in plastid membranes, and that it lies at the intersection of many redox pathways. These include the desaturation reactions of carotenoid biosynthesis and chlororespiration (15). Reminiscent of AOX, PTOX also appears to serve as a “safety valve” for the dissipation of excess electron flow, e.g., during stress (16-18).

AOX and PTOX are members of the DOX (non-heme diiron carboxylate quinol oxidase) family of proteins (19-24). By analogy to crystal structure determinations of non-plant members of this family, it has been proposed that the diiron centers of AOX and PTOX
are coordinated by four carboxylate and two His residues on a four helix bundle (Figure 1B) (21-23). Sequence alignments have revealed that the six putative Fe-binding residues are highly conserved in the sequences of all AOX and PTOX proteins examined to date. In addition, nearly all PTOX enzymes contain a 16 amino acid domain near the C-terminus that is highly conserved, but that is not found in AOX. Curiously, this sequence corresponds precisely to Exon 8 of the gene.

We previously used PTOX as a model to test the functional significance of the conserved Fe-binding and Exon 8 sequences (10). These experiments were facilitated by the availability of two important tools: 1: an in vitro activity assay, developed by Josse and coworkers (12, 14); and 2) null alleles of the Arabidopsis immutans (im) mutant, which made in planta mutagenesis experiments possible. im plants are green- and white-sectored due to action of a nuclear recessive gene (25, 26). The white sectors contain abnormal, photooxidized plastids as a consequence of a lack of colored carotenoid production, while the green sectors contain morphologically-normal chloroplasts. When transformed with a wild type IM sequence, im plants revert to an all-green phenotype. Mutagenized copies of IM can thus be tested for their ability to normalize the im variegation and restore a wild type appearance. The in vitro and in planta experiments showed that the six amino acids that bind Fe do not tolerate change, even conservative ones, and that the Exon 8 domain is required for PTOX activity and stability (10).

In this manuscript we examine 14 other amino acids that are perfectly-conserved, or nearly so, in the sequences of all AOX and PTOX reported to date: L135, P142, A139, H151, E171, N174, L179, Y212, P216, A230, Y234, D287, R293, D295 (numbering refers to the Arabidopsis PTOX sequence—see Figure 1B). Most of these residues are predicted to reside
in close proximity to the six Fe-binding sites. In this paper we test the functional significance of these sites by in vitro and in planta alanine scanning mutagenesis, and report that very few of these sites are absolutely essential for activity. In addition, some mutant enzymes are defective in vitro, but are able to complement im. RNAi, antisense and co-suppression experiments showed that transgenic plants with less than 3% of wild type PTOX levels produce normal-appearing plants. Considered together, the data suggest that PTOX is normally in excess, especially during the process of thylakoid formation early in leaf development.

MATERIALS AND METHODS

Plant growth – The plants in this paper included wild type Arabidopsis (Columbia ecotype), the spotty allele of im (also in Columbia) (8), and transgenic im plants (described below). The plants were germinated and grown at 22°C under conditions of continuous illumination. The wild type plants were maintained at 100 μmol m⁻² s⁻¹. However, spotty is light sensitive and therefore was germinated under low light (15 μmol m⁻² s⁻¹ for 5 days) before transfer to normal light (100 μmol m⁻² s⁻¹).

Site-directed mutagenesis in vitro - An IM cDNA lacking the N-terminal chloroplast targeting sequence (8) was cloned into the BamHI and NheI sites of the E. coli expression vector pET-11a (Novagen, Madison, WI). The QuikChange™ Site-Directed Mutagenesis protocol (Stratagene, LaJolla, CA) was used to generate mutations; the mutations were confirmed by sequencing. Following standard notation, the mutant clones are designated by the amino acid that was altered, its location in the A. thaliana IM sequence and the amino acid to which it was changed (e.g., L135A means Leu¹³⁵ was changed to Ala).
The following primers were used to introduce mutations into 14 conserved amino acids residues of AOX and IM:

L135A: 5’-gcaaggttcttttgctgagacgc-3’ 5’-gctgtctcagcaacaagaaccttg-3’
A139G: 5’-cttgagacaatttgtagagtgcctttg-3’ 5’-gcaaaaaagccactctacaatttgct-3’
P142A: 5’-caattgcctagatgcttgtgc-3’ 5’-cgcaaaataagccactctagcaattt-3’
H151A: 5’-gtctgtgctagctatagctatcag-3’ 5’-ccaaaggtctcatacatagccatg-3’
E171A: 5’-gtacactttgctgagcttgtaa-3’ 5’-caatttcattccagctcttagc-3’
N174A: 5’-gagagctgggctgaaatgcccaagtc-3’ 5’-caaaataaggcactctagcaattt-3’
L179A: 5’-gaaatgcatcacgcgctcataatggaag-3’ 5’-cttccattatgagcgcgtgatgc-3’
Y212A: 5’-gtgtcttgctatcttacagccatag-3’ 5’-cattctagggcttaagatagcc-3’
P216A: 5’-gtatatcttaagcctagctatcagtcacag-3’ 5’-gtgtcttataactcagcc-3’
A230G: 5’-gtggagagtccagtgcagcc-3’ 5’-ataaggtctcataatcagc-3’
Y234A: 5’-gcatatgagactgtctcataatcagtc-3’ 5’-gagaaatttatcagcagtctcat-3’
D287A: 5’-gaaaatctatacgctgtgtttgtc-3’ 5’-gttcacaaacacgcagttg-3’
R293A: 5’-gtgagactgctttctattcagtc-3’ 5’-gtgtcttctcataatcagtc-3’
D295A: 5’-gtgacataagagatgcagccagacacac-3’ 5’-gtgtcttctcagcactcagtc-3’

Site-directed mutagenesis in planta - Procedures for in planta mutagenesis have been described (10). In brief, mutations were introduced (as above) into full length IM cDNAs, and the mutant cDNAs were cloned behind the CaMV 35S promoter in the binary vector pBI121. The constructs were transferred into Agrobacterium tumefaciens and introduced into im plants (spotty allele) by the floral dip method (27). Kanamycin-resistant seedlings were selected at the T1 generation on plates containing 1X MS salts (pH 5.7), 1% sucrose and 50 μg ml−1 kanamycin. PCR and Southern blotting methods were performed to verify that
the plants were transformed. Phenotypic analyses and protein analysis were conducted on T2 generation plants.

**Generation of antisense and co-suppression plants** - Full length wild type IM cDNAs (the same as used for the *in planta* site-directed mutagenesis experiments) were cloned in the forward and reverse orientations behind the CaMV 35S promoter in the binary vector pBI121. The same procedures were used as described above for plant transformation. Western blotting was performed to identify antisense and cosuppression lines with reduced levels of the IM protein in T2 generation plants; these procedures have been described (10).

**PTOX activity assays** – We have previously described methods to estimate *in vitro* PTOX activity based on procedures described by Josse et al. (12, 14). In brief, mutant constructs were transformed into *E. coli* strain BL21-DE3, and protein expression was induced by the addition of 1.6 mM IPTG (final concentration). The cells were then lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM PMSF and 0.3% protease inhibitor cocktail (PIC). The membranes were collected by ultracentrifugation (200,000g for 2 hours at 4°C), and the concentration of proteins in the pellet was measured using the Bio-Rad protocol (Hercules, CA) with BSA as a standard. Oxygen consumption was monitored at 25°C with a Clark O2 electrode (Hansatech, Norfolk, England) using 100 – 200 µg of membrane protein in a 1.5 ml reaction mixture that contained a final concentration of 50 mM Tris-maleate (pH 7.5), 10 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 0.2 mM decyl-plastoquinone (2,3-methyl-5decyl-1,4-benzoquinone) (Sigma, St. Louis, MO). The reactions were initiated by the addition of NADH (1 mM final concentration), followed by KCN (3 mM final concentration) and *n*-propyl gallate (3,4,5-tri-hydroxy-benzoic acid-*n*-propyl ester) (0.5 mM...
final concentration), all from Sigma. Reactions were followed for a total of 8 min: KCN was added 1 min after initiating the reaction with NADH; n-PG was added after 6 min.

**Isolation and detection of proteins** - Proteins were isolated from partially-purified chloroplasts by previously described procedures (28). In brief, leaves from four week old Arabidopsis seedlings were homogenized in 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 the filtrate was centrifuged at 2600g for 5 min. The pellet was resuspended in 10 mM 3-(N-morpholino) propane-sulfonic acid (MOPS) (pH 8.0) then centrifuged at 10,000g for 10 min. The resulting pellet was resuspended in 0.33 M sorbitol, 5 mM MgCl₂, 50 mM HEPES (pH 8.0), and the chlorophyll concentration of the suspension was determined (28).

Procedures for Western blotting have been described (10). In brief, 5 μg protein (for *E. coli* samples) or an amount of protein corresponding to 5 μg chlorophyll (for chloroplast samples) were electrophoresed through 12.5% SDS polyacrylamide gels, and the proteins were transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody generated to the Arabidopsis PTOX protein (1:3000 dilution) (16) and the proteins were visualized using the ECL immunodetection procedure (Pierce, Erembodegem, Belgium).

**RESULTS**

*Identification of conserved residues in PTOX and AOX* - Figure 1A shows that AOX and PTOX have two structural features in common: both have an N-terminal transit peptide (TP) that targets the protein to the organelle (mitochondria in the case of AOX and chloroplasts in
the case of PTOX); and both have a "DOX" domain, which comprises a four-helix bundle that includes the catalytic site (22-24). The active site binds the quinol substrate and is composed, in part, of six amino acids that serve as ligands for two iron atoms (E136, E175, H178, E227, E296 and H299) (Figure 1B). AOX and PTOX also have unique structural domains: AOX contains a "dimerization domain" (D-domain) that has been implicated in AOX-AOX dimer formation (29), and PTOX contains a 16 amino acid (Exon 8) domain near the active site that is essential for protein stability and activity (10).

When the present experiments were initiated (in Fall, 2003), 7 PTOX and 20 AOX protein sequences were available for comparison. 20 residues were found to be perfectly-conserved among these sequences, and these 20 became the focus of our structure/function studies. The conservation of 13 of these sites was subsequently noted by Finnegan et al (24) and McDonald et al (30). Six of the 20 residues are putative Fe-binding sites, and our results of in vitro and in planta mutagenesis of these six have been recently reported (10). The present manuscript describes mutagenesis studies on the other 14 residues. It might be noted that as more AOX and PTOX sequences have become available, the 20 residues remain the most highly conserved sites among 124 AOX and PTOX sequences in the NCBI non-redundant protein database (as of Spring 2006): five of the 20 residues are still perfectly-conserved; 11 are conserved in 123 out of the 124 sequences; and four residues have three exceptions (Figure 1B).

Mutagenesis of 14 conserved residues in vitro - To test the functionality of the 14 residues, we conducted site-directed mutagenesis using an in vitro PTOX activity assay developed by Josse et al. (12, 14); this assay was used by us in our functional analyses of the six Fe-binding sites (10). In brief, this assay involves the measurement of O2 consumption using
membranes from *E. coli* transformed with various mutant *IM* constructs. Prior to measurement, the membranes are treated sequentially with NADH, KCN and *n*-propyl gallate (*n*-PG). The rationale is that NADH serves as an electron donor to quinone (via a membrane-bound NADH dehydrogenase), and electrons are then transferred to molecular oxygen via either PTOX or the cytochrome pathway. Flux is regulated by KCN, which inhibits the cytochrome pathway (but not PTOX activity), or by *n*-propyl gallate (*n*-PG), which inhibits PTOX but not the cytochrome pathway. In short, O₂ consumption occurs by the cytochrome pathway when *n*-PG is added to the membranes, but by PTOX when KCN is added. Addition of both inhibitors abolishes O₂ consumption.

For our experiments we performed alanine scanning mutagenesis on 12 of the 14 residues; the two conserved alanine residues (A139 and A230) were mutated to glycine. Figure 2A shows that wild type PTOX has an average KCN-resistant O₂ consumption activity of 115 nmol O₂ min⁻¹ mg-protein⁻¹ (lane 2); this activity is negligible in an empty vector control (~3 nmol O₂ min⁻¹ mg-protein⁻¹) (lane 1). Eight of the mutants have KCN-resistant O₂ consumption rates similar to the wild type protein (A139G, E171A, N174A, L179A, P216A, A230G, D287A, R293A), but O₂ consumption is nearly abolished in five of the mutants (L135A, H151A, Y212A, Y234A and D295A). One of the mutants, P142A (lane 5), has a moderate reduction in O₂ consumption (~60% of wild type levels). We conclude that five of the conserved amino acids are essential for activity, and that a sixth is important for optimal activity.

To examine whether the severe decreases in KCN-resistant O₂ consumption are due to poor expression and/or instability of the mutant proteins, PTOX abundance was assayed by Western blot analysis of membrane proteins from the various transformants using a
polyclonal IM antibody (10, 16). Fig 2B shows that mutant and wild type PTOX accumulate to similar levels in E. coli (lane 2); the empty expression vector, as anticipated, lacks PTOX. We also found that PTOX is localized in the membrane (versus soluble) fractions in all the samples, consistent with the idea that the various mutations do not compromise protein incorporation into the E. coli membrane (data not shown). One other possibility for the lack of expression of some of the constructs is that the proteins fail to fold properly. We have previously discussed the evidence that makes this unlikely (10). One is that the 3-D structures of DOX proteins appear to be quite stable and resistant to alterations in charge or mobility of side chains. Taken together, the data indicate that the mutant PTOX proteins are expressed and stable at wild type enzyme levels, and that reductions in O₂ consumption are due to reductions in PTOX activity.

**Mutatgenesis of 14 conserved amino acid residues in planta** - Mutants that lack PTOX (e.g., im) are a powerful tool to gain insight into structure/function relationships of PTOX and AOX (10). This is because of the high degree of structural similarity of their DOX domains. The phenotype of im will be briefly reviewed because our in planta assay relies on the ability of mutant PTOX sequence to alter the variegation phenotype of im.

**Phenotype of im** - As mentioned above, Arabidopsis im mutants have a light-sensitive green-and white-variegation—enhanced light intensities promote white sector formation (25, 26, 31). Early observations showed that the white im sectors accumulate phytoene, a colorless C₄₀ carotenoid precursor, indicating that im is defective in the phytoene desaturase (PDS) step of carotenogenesis (26). Current thinking is that this accumulation is a consequence of the fact that PTOX is a component of a redox chain that functions in phytoene desaturation in the thylakoid membrane (32). In this chain, electrons are transferred from phytoene to the
plastoquinone (PQ) pool (via PDS), and thence to molecular oxygen (via PTOX) or to the cytochrome b6/f complex. This hypothesis has received considerable experimental support (reviewed in 15, 32).

Mechanism of im variegation - IM is a single copy gene in all organisms examined (10), and thus an intriguing question is why some cells are green and others white, against a mutant background. Consistent with the above molecular and biochemical observations, we hypothesize that a lack of PTOX (as in immutans) results in over-reduction of the PQ pool under restrictive conditions (e.g., high light); this has been demonstrated (17). This over-reduction prevents further transfer of electrons to the PQ pool and, consequently, a blockage of the carotenoid pathway at the PDS step (the first of two desaturations in the carotenoid pathway). This results in an accumulation of phytoene and a lack of production of downstream, colored (photoprotective) carotenoids. Carotenoids quench triplet chlorophyll and toxic oxygen radicals, and in their absence, photooxidized, white plastids are generated under restrictive light conditions. We further hypothesize that PTOX might be particularly important as an electron sink during early chloroplast biogenesis when undifferentiated proplastids in the leaf meristem develop into photosynthetically-competent chloroplasts, and components of the photosynthetic apparatus are synthesized and assembled. During this stage, some plastids lack a threshold of photoprotective activities (white plastids), while others, even in the absence of PTOX, have a threshold and develop into functional chloroplasts (8). We propose that green and white sectors in developing and mature leaves are primarily a reflection of large-scale differences in restrictive and permissive conditions perceived by the developing leaf, e.g., regions of the meristem that are shaded might translate
into green patches in the mature leaf, whereas regions that perceive high light translate into white patches.

*Mutagenesis in planta* - Taking advantage of null alleles of *im*, we tested the impact of each of the 14 mutations described above on PTOX activity *in planta*. Wild type *IM* cDNAs and *IM* cDNAs with single mutations in each of the 14 residues were generated and transformed into *im*. Full-length cDNAs were used to assure proper targeting to the plastid; the constructs were driven by the CaMV 35S promoter. 12 independent transgenic lines were selected in the T1 generation for each mutant construct (i.e., a total of 12 x 14 = 168 independent transgenic lines were examined). Each of these lines had intact inserts, as determined by PCR and Southern blotting using primers or a probe specific for the Bar gene. The T1 plants were selfed, and Western blotting was carried out to determine the abundance of PTOX in the 168 T2 generation lines. Mutants with similar levels of PTOX (Figure 3B) were selected for phenotypic comparisons (Figure 3A).

Figure 3A shows that *im* plants transformed with the empty BI121 binary vector resemble non-transformed *im* ("pBI121"), whereas transformation with wild type *IM* produces all-green leaves ("WTIM"). The 14 mutants fell into three categories:

i). Transformation of *im* with nine of the mutant DNAs generated all-green transgenics: A139G, P142A, E171A, N174A, L179A, P216A, A230G, D287A, and R293A. These mutants were able to complement the mutant phenotype. All of these mutants had normal *in vitro* activities except P142A, which had an ~40% reduction. This suggests that, with the exception of P142A, these sites are not important for PTOX activity. The P142A results suggest that a defective enzyme with only 60% of normal activity is able to confer all the activity that is needed for normal function *in planta*. 
ii). Transformation of \textit{im} with three of the mutant constructs (L135A, H151A, and Y234A) gave rise to variegated plants, but the extent of variegation was significantly less than found in \textit{im}—i.e., these plants were primarily green. These mutants had negligible \textit{in vitro} activities. This suggests that these sites are important for PTOX activity, but that not much activity is required for normal function \textit{in planta}.

iii). Y234A and D295A produced transgenic \textit{im} plants that resembled \textit{im} and they had negligible \textit{in vitro} activities. This suggests that these sites are essential for PTOX activity.

\textit{RNAi, antisense and co-suppression plants} - The data in Figures 2 and 3 were based on samples (\textit{E. coli} or Arabidopsis) with similar amounts of PTOX protein. We were next interested in determining the phenotypes of plants with different amounts of the wild type enzyme. For this purpose we carried out a titration experiment in which we modulated PTOX levels using RNAi, antisense and co-suppression technologies. Figure 4A shows a representative sample of the transgenics; wild type and a null allele of \textit{im} are controls.

Figure 4B shows that lines with greater than \textasciitilde 3% of wild type PTOX levels have a normal phenotype and growth habit. By contrast, the RNAi line (in which PTOX is not detectable) is virescent (delayed greening). All the leaves of the RNAi plants are yellow-green during expansion, but turn all-green after expansion is complete. At flowering the leaves and plants are nearly normal-sized. Interestingly, we failed to identify variegated plants among the various transgenic populations, despite the fact that plants could be found with no detectable PTOX protein accumulation (such as the RNAi line). We conclude that PTOX is normally in excess of the amount required for normal chloroplast development and function, at least under the growth conditions used.
DISCUSSION

Evolution of AOX and PTOX - It is thought that α-proteobacteria and cyanobacteria are the ancestors of mitochondria and chloroplasts, respectively (33, 34). These were likely unique endosymbiotic events. During subsequent evolution, it is thought that most genes were lost or transferred from the symbionts to the host genomes, leaving behind organelle genomes that represent remnants of their former selves. In the case of genes transferred to the nucleus, many subsequently acquired N-terminal targeting signals for post-translational import back into the organelle. Phylogenetic studies have revealed that genes for AOX and PTOX have a prokaryotic origin and, intriguingly, that they diverged prior to the endosymbiotic events that gave rise to mitochondria and chloroplasts (24, 30, 35, 36). It has been suggested that their absence in lineages such as animals is due to gene loss in ancestral species (24).

Given the evolutionary distance between AOX and PTOX, it is striking that certain residues are perfectly-conserved, or nearly so, among the 124 PTOX and AOX sequences in the databases as of Spring 2006, with most differences arising in those taxa most distantly related to higher plants -- TcAOX, SyPTOX, PmPTOX, and BnPTOX (protists, cyanobacteria, and fungi, respectively). For instance, 9 of the 20 conserved residues in Figure 1B are not conserved in the animal parasite protozoa Trypanosoma cruzi AOX (TcAOX). This includes three of the six Fe-ligands, which suggests that AOX is capable of a high degree of species-specific flexibility in its diiron reaction center. In fact, excluding TcAOX from Figure 1B, the number of perfectly-conserved residues jumps from 5 to 12.

Classes of mutants - In the present experiments we tested the functionality of 14 of the 20 conserved residues using both in vitro and in planta mutagenesis procedures. In an earlier
paper we examined the other six of these 20 sites (the putative Fe-binding ligands) and found that they are essential for activity *in vitro* and *in planta*; they do not tolerate change, even conservative ones. In this report we found that the 14 residues fall into three classes:

I. Essential for activity *in vitro* and *in planta*: Y234 and D295

II. Not important for activity *in vitro* and *in planta*: A139, E171, N174, L179, P216, A230, D287, and R293.

III. Important but not essential for activity: P142, L135, H151, and Y212.

Classes I and II are relatively straightforward to explain: mutant enzymes with negligible activity *in vitro* are not able to complement im (and are thus essential for activity), whereas mutant enzymes with wild type activity *in vitro* are able to complement im (and are thus non-essential for activity). On the other hand, it is more difficult to explain the class III sites. Class III mutant enzymes have defective activities *in vitro* but are able to complement *im*, at least in part. Comparisons of activities in the *in vitro* and *in planta* experiments were based on *E. coli* and Arabidopsis with the same amount of PTOX protein, so the class III mutants cannot be explained by a higher abundance of the mutant enzyme in some samples versus the others. An alternative hypothesis is that *E. coli* and thylakoid membranes differ in composition, and thus the mutant enzymes interact with different membrane components: this interaction inhibits activity in *E. coli* but not in thylakoids. Still a third hypothesis is that the enzymes are defective to a similar extent in *E. coli* membranes and thylakoids, but that only low levels of activity are required to produce transgenic *im* plants that are all-green or nearly so. Consistent with the latter possibility is the titration experiment (Figure 4), which shows that normal-appearing plants can be produced with only ~3% of wild type PTOX. This suggests that PTOX is normally in excess, at least under ambient Arabidopsis growth
conditions (22°C, continuous illumination at 100 μmol m⁻²s⁻¹). It would be interesting to test the downregulated PTOX plants under stress conditions. Regardless, given our hypothesis about the mechanism of *im* variegation, it is likely that PTOX excess is especially important during early chloroplast biogenesis when thylakoid membranes are forming.

*Function of conserved active site amino acids-*

Given the three classes of mutant enzymes, the question arises about the functions of the 20 conserved residues. In the AOX/PTOX structural model of Andersson and Nordlund (21) and Berthold et al. (22), the diiron site resides within the hydrophobic crevice of a four-helix bundle, and the protein is anchored in the membrane by a fifth, short alpha-helical domain (i.e., AOX and PTOX are interfacial membrane proteins). The functions of the conserved sites will be considered by each helix in turn.

*Helix 1: L135, E136, A139, P142 & H151*

Helix one contains one of the six Fe ligands, the perfectly-conserved E136 (see Figure 1B). The importance of this site has been tested experimentally by mutagenesis (10). Immediately flanking E136 are the perfectly-conserved L135 and A139, as well as P142, which is different only in TcAOX (where the Pro is changed to Ala). Further downstream is the perfectly-conserved H151.

It has been proposed that L135, A139 and P142 (non-polar, hydrophobic R groups) are important for the structure of the hydrophobic crevice, while the positively-charged H151 might interact with phosphate groups on the membrane (21) or, alternatively, be involved in substrate binding or electron transfer (22). Given the high degree of conservation of these sites, it is surprising that our functional analyses revealed that these sites are either not important for activity (A139) or are important but not essential for activity (L135, P142,
H151), at least when these amino acids are converted to Ala or Gly (for A139). We surmise that if L135 and A139 play a role in the structure of the hydrophobic crevice, substitution with Ala or Gly (respectively) might not be a dramatic enough change to compromise function, since both have small, uncharged R groups. On the other hand, the Pro to Ala change in P142A might be expected to perturb protein conformation. This would be consistent with the \textit{in vitro} assays (a 40% reduction in activity), but not with the \textit{in planta} results, where normal-appearing plants were recovered. As discussed above, this might be because the P142A mutant interacts differently with \textit{E. coli} versus thylakoid membranes, or perhaps because only low levels of PTOX activity are necessary for normal growth.

Finally, the perfectly-conserved H151, when converted to Ala, knocked out enzyme activity \textit{in vitro}. This is a change of a positively charged R group with an uncharged group, and in keeping with the high degree of conservation of this site. Hence, the fact that near-normal \textit{im} transgenics were produced by H151A mutants was surprising. Again, this might suggest that a positive charge is important at this site in \textit{E. coli} membranes, but not in thylakoids, or alternatively, point toward the fact that normal growth is compatible with low levels of PTOX activity.

\textit{Helix 2: E171, N174, E175, H178 & L179}

Helix two contains two of the six Fe ligands (E175 & H178); these are perfectly-conserved, except in TcAOX (where E175 is His) and in EnAOX (where H178 is Phe). The importance of E175 and H178 has been demonstrated experimentally by mutagenesis in PTOX and AOX (10, 37-40). Immediately flanking the EXXH sequence are the residues E171, N174 and L179. E171 is not conserved in SyPTOX, PmPTOX, and TcAOX (it is a Gln in these three sequences), while N174 is not conserved in TcAOX (it is a Ser).
Moreover, L179 is not conserved in SyPTOX (it is a Glu). In the 3-D structure proposed by Berthold et al (22) and Andersson and Nordlund (21), N174 is close to the Fe-binding motif in helix 4 (EXXH), and they proposed that N174 interacts with E296 or H299 via a hydrogen bond to stabilize the four-helix bundle and/or to fine-tune Fe coordination. This notion has been supported by the phylogenetic studies of Finnegan et al (24). L179 has a hydrophobic R group, and thus its conservation might suggest that it is important in defining the structure of the hydrophobic crevice. The role of E171 is not clear in the model, but the negatively charged group might facilitate electron transfer or stabilization of the pocket structure.

Despite the conservation of E171, N174 and L179, our *in vitro* and *in planta* experiments showed that they are not important for activity (class II sites). This might have been because the changes were not dramatic enough, at least in the case of L179A, since Leu and Ala both have hydrophobic R groups. However, the E171A substitution seems substantial, at least *a priori* (a large, negatively charged R group for a small, nonpolar one). The reason for the conservation of these three residues remains to be determined.

*Helix 3: E227 & Y234 -- The Nakamura E(X)_6 Y motif*

In their structure/function studies of the *Trypanosoma vivax* AOX, Nakamura et al. (2005) noted that the helix 3 motif, E(X)_6 Y, is perfectly-conserved in all AOX and PTOX proteins (41). This motif extends from E227 to Y234 in the PTOX sequence (Figure 1B). As more sequences have become available, the conservation of this motif remains near-perfect, the only exception being TcAOX. E227 is one of the Fe-ligands (10), and the Berthold et al (22) and Andersson and Nordlund (21) models predict that Y234 is near the di-iron site.
Our *in vitro* and *in planta* studies confirm the essential nature of E227 and Y234 (Figures 2, 3 and reference 10). They are also in accord with the studies of Nakamura et al. (2005) showing that conversion of E227 and Y234 to Ala abolishes activity of the trypanosome AOX in an *E. coli*/ΔhemA activity assay. The reason for the importance of E227 is clear: it serves as an Fe-ligand (10, 21, 22). The reason for the essential nature of Y234 is less clear, but given its location, two functions that have been ascribed to Tyr residues are potentially applicable to this site. One is in electron transport via the hydroxyl group (42, 43), and the other is quinone binding via the aromatic ring (44, 45). The conversion of Y234 to Ala does not let us discriminate between these possibilities. However, Albury *et al.* (40) substituted Y275 of the *Sauromatum guttatum* (voodoo lily) AOX (which corresponds to Y234) with Phe to test the functional significance of the OH group (Phe lacks the hydroxyl group but retains the aromatic ring). They found that the mutant protein (Y275F) has no activity when tested in the *E. coli*/ΔhemA strain, suggesting that Tyr275 is essential because it is involved in electron transfer. Our data are consistent with this interpretation.

*Helix 3: Y212, P216 & A230*

In addition to the conserved E(X)₆Y motif, Nakamura *et al.* (2005) noted that Y199 of the *Trypanosoma vivax* sequence is perfectly-conserved (corresponds to Y212, Figure 1B); in the most recent database search, 121 of 124 PTOX and AOX sequences have Tyr at this site. Nakamura *et al.* (2005) found that substitution of Y199 with Ala reduced the activity of the trypanosome AOX approximately 50%, using the *E. coli*/ΔhemA activity assay. On the other hand, Albury *et al.* (40) found that substitution of the corresponding Tyr in the voodoo lily enzyme with Phe (Y253F) did not impact activity, again using the *E. coli*/ΔhemA assay. We found that the *in vitro* activity of the Y212A mutants was nearly abolished, but that the
Y212A transgenics were only slightly variegated, indicating that the mutant enzyme was able to restore a large measure of wild type function to thylakoids. Again, this is consistent with the notion that only low levels of PTOX activity are required in planta (i.e., the enzyme is normally in excess), or perhaps that Y212 is essential in E. coli membranes but not in thylakoids.

Whereas the differences in the in vitro assays between Albury (40) and Nakamura (41) could be due to the types of substitution made, we do not understand the qualitative difference in the in vitro activities between Nakamura et al. (2005) and ourselves, especially since both relied on an E. coli-based assay. This could point up the fact that AOX and PTOX are not structurally the same (Figure 1A), and that one should exercise caution in extrapolating results from one enzyme to the other. Nevertheless, the conservation of this site might suggest that Y212 is analogous to Y234 and functions in either redox chemistry or substrate-binding.

P216 and A230 are highly conserved (Figure 1B) but have not previously been examined functionally. P216 is changed to His in SyPTOX, Arg in MeAOX, and Leu in CgAOX, while A230 is changed to Ser in TcAOX. We found that P216 and A230 are unimportant for activity when converted to Ala and Gly, respectively. As with P142 (Helix 1), a Pro to Ala change (P216A) might be expected to perturb protein conformation, but if this is important, we could not detect it in our assays. Regarding A230, altering this residue to Gly might not have been a significant enough substitution to observe a change. The reasons for the high degree of P216 and A230 conservation remain to be resolved.

*Helix 4: D287, R293, D295, E296 & H299*
Helix four contains two of the six iron ligands (E296 & H299). As with the EXXH sequence on Helix 2, the helix four EXXH sequence is perfectly-conserved except that E296 is a Val in TcAOX and H299 is an Ile in wheat PTOX. The functional importance of E296 and H299 has been demonstrated by mutagenesis both in PTOX and AOX (10, 38, 39, 46), but D287, R293, D295 have not yet been examined. The alterations of these latter three sites are predicted to be substantial, since charged R groups were substituted for Ala. Despite this, we found that these changes do not affect the activity of the D287A and R293A mutants, making the reason for their conservation unclear. In contrast, the perfectly conserved D295 was one of only two residues we identified that are absolutely essential for activity both in vitro and in planta. The 3-D structural model predicts that D295 is close to E175 and H178 (Fe ligands in Helix 2). This is analogous to the situation in which N174 (helix 2) is close to the EXXH motif in helix 4, and suggests that D295 interacts with E175 and/or H178 via hydrogen bonding to stabilize the active site structure or Fe atom coordination.

Summary - Our previous mutagenesis studies showed that all six of the Fe-ligands are essential for activity in vitro and in planta. However, the present studies were only able to identify two more residues in this class, with the majority having no impact on either in vitro or in planta activity. However, it is possible that appropriate substitutions were not made. Another possibility is that some of the mutant plants were not grown under optimal conditions -- e.g., the mutant enzyme might function normally under ambient conditions but not under stress conditions. The same caveat holds for the titration experiments. Whereas these experiments showed that PTOX is in excess under ambient growth conditions, the same might not be true for stress conditions, where PTOX is thought to be important as a “safety valve” to dissipate excess electron flow. This would follow on the landmark flux control
studies of Stitt and colleagues using antisense rbcS plants of tobacco in the early 1990's (reviewed in 47). They found that plants normally have a reservoir of Rubisco (Rubisco is not limiting for photosynthesis), but that under conditions of substrate or nutrient limitation this reservoir becomes depleted, and Rubisco is strictly limiting for photosynthesis. The present studies thus set the stage for further structure/function studies on PTOX.

REFERENCES


FIGURE LEGENDS

Figure 1. Conserved residues in AOX and PTOX.

A. Structures of AOX and PTOX. Both proteins have an N-terminal organelle targeting sequence (TP, transit peptide). AOX has a dimerization domain (D-domain) (not present in PTOX), whereas PTOX has an Exon-8 sequence (absent in AOX). The DOX domain is shown in gray.

B. Sequence alignments in Fall 2003 revealed the presence of 20 perfectly-conserved residues in comparisons of the 27 AOX and PTOX sequences that were available at that time. By Spring 2006, 124 AOX and PTOX sequences were available in the
NCBI non-redundant protein database. These sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw) and the 20 amino acids were still the most highly conserved among the 124 sequences. The figure shows the 20 amino acids: 5 of the residues are perfectly conserved (indicated by an arrow); 11 residues are conserved in 123 of the 124 sequences (indicated by "*"); and four residues are conserved in 121 of the 124 sequences (indicated by "**"). The six iron binding sites are indicated by darkened circles and the location of the four helices is shown. The exceptions are the following: P142 (A in SyPTOX), N174 (S in TcAOX), E175 (H in TcAOX), H178 (F in EnAOX), L179 (E in SyPTOX), E227 (G in TcAOX), A230 (S in TcAOX), Y234 (W in TcAOX), D287 (S in TcAOX), E296 (V in TcAOX), and H299 (I in TaPTOX), E171 (Q in SyPTOX & PmPTOX, TcAOX), Y212 (F in TcAOX, T in SyPTOX, W in BnPTOX), P216 (H in SyPTOX, R in MeAOX, L in CgAOX), and R293 (C in CrPTOX, A in PmPTOX, V in SyPTOX).

MeAOX, uncultured bacterium MeDeBAC49C08 AOX, gi: 67906549; BnPTOX, alga Bigelowiella natans PTOX, gi: 32307546; EnAOX, Fungi Emericella nidulans AOX, gi: 24061751; TaPTOX, wheat Triticum aestivum PTOX, gi: 9837152; CrPTOX, Chlamydomonas reinhardtii PTOX, gi: 20149254; TcAOX, parasitic protozoa Trypanosoma cruzi strain CL Brener AOX, gi: 71665747; PmPTOX, cyanobacteria Prochlorococcus marinus subsp. Pastoris str. CCMP1986 PTOX, gi: 33633836; SyPTOX, Cyanobacteria Synechococcus sp. WH8102, gi: 33632590; CgAOX, Fungi Chaetomium globosum CBS 148.51 AOX, gi: 88181006

Figure 2. Mutagenesis of 14 conserved residues in vitro

A. The oxygen consumption rates were determined from the slopes of O2 traces following the addition of 1 mM NADH, 3 mM KCN, and 0.5 mM propyl gallate (PG)
PTOX activity is defined as the oxygen consumption rate in the presence of 3 mM KCN minus the oxygen consumption rate in the presence of 0.5 mM n-PG. Each rate is the average of four independent measurements (+/- SD). 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:
- Lane 3, L135A
- Lane 4, A139G
- Lane 5, P142A
- Lane 6, H151A
- Lane 7, E171A
- Lane 8, N174A
- Lane 9, L179A
- Lane 10, Y212A
- Lane 11, P216A
- Lane 12, A230G
- Lane 13, Y234A
- Lane 14, D287A
- Lane 15, R293A
- Lane 16, D295A

B. Total membrane proteins were isolated from transformed *E. coli*, and equal amounts were electrophoresed through 12.5% SDS-PAGE gels, then transferred to nitrocellulose membranes. The membranes were treated with an antibody to *Arabidopsis* PTOX and visualized by the ECL system. Lanes are as in (A).

**Figure 3.** Mutagenesis of 14 conserved residues *in planta.*

A. The same mutations 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*). At least eight transformation events per construct were examined; T2 generation seedlings are shown. All plants were grown for three-to-four weeks under continuous light (100 μmol m⁻² sec⁻¹) after initial low light growth.

B. Western blotting analyses were conducted using 5 μg of chlorophyll per lane from chloroplast membranes of partially-purified plastids using an antibody to *Arabidopsis* PTOX. The proteins were visualized by the ECL system.
Figure 4. PTOX RNAi, antisense and co-suppression plants

A. Representative RNAi, antisense and co-suppression plants (T2 generation) are shown. Kanamycin-resistant seedlings were scored by PCR and Southern blotting for the presence of the kanamycin gene (*NPTII*).

B. Western immunoblot analyses were conducted using 5 ug of chlorophyll per lane of partially-purified chloroplast membranes from WT leaves and leaves of RNAi, antisense and co-suppression plants. The filters were probed with an antibody to Arabidopsis PTOX. The numbers are the relative amount of PTOX compared to WT (100%). ND = not detectable.
FIGURE 1
FIGURE 2
FIGURE 3

FIGURE 4
CHAPTER 4. PROTEOMIC ANALYSIS OF ARABIDOPSIS IMMUTANS MUTANT

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Aigen Fu and Steven Rodermel

ABSTRACT

The Arabidopsis immutans mutant is variegated due to a nuclear recessive gene mutation. The mechanism of variegation development under a uniform mutant genetic background is poorly understood. Here, a comparative proteomic approach was applied to investigate the green and white sectors of im. Out of 500 spots on 2-D gels that were analyzed by MALDI-TOF, five (corresponding to two different proteins) showed a 3-fold increase in im green tissues compared to wild type. No spot was significantly down-regulated. Such a small difference in proteome profiles is consistent with the observation that the green sectors are phenotypically normal. In im white tissues, 45 spots were down-regulated and 86 spots were up-regulated by 3-fold or more compared to wild type. MALDI-TOF analysis showed that all 15 down-regulated proteins are chloroplast proteins that function in photosynthesis. This is consistent with the idea that the white sectors are not photosynthetic, and that photodamage is primarily limited to plastids. On the other hand, the 46 up-regulated proteins in im white tissues included 20 chloroplast proteins, 8 mitochondrial proteins, and 18 cytosolic proteins. The nature of the up regulated proteins suggests that there might be a higher respiratory activity in white sectors, and that the white cells are induced to change various biological pathways to adapt to photodamage. Norflurazon, an herbicide that inhibits carotenoid biosynthesis at the phytoene desaturase (PDS) step, has a similar effect on gene expression as the im mutation, which indirectly affects the PDS step. Only 9 spots
INTRODUCTION

Carotenoids are important pigments present in all plastids, and they are integral and essential components of plastid membranes (Sandmann, 2001). Carotenoids are involved in a number of processes required for plastid structure and function, including light harvesting (Fromme et al., 2001), thylakoid membrane stabilization (Havaux, 1998), and photoprotection (Demmig-Adams et al., 1996). Sunlight is used by plants to drive photosynthesis, which converts carbon dioxide into carbohydrates. Excess light captured by chlorophyll can generate triplet chlorophyll and singlet oxygen. If not quenched, this energy can be transferred to oxygen, generating reactive oxygen species (ROS). The accumulation of ROS can result in photo-oxidization of the contents of the chloroplast (Niyogi, 2000; Mittler, 2002). Carotenoids protect chloroplasts against photo-oxidative damage by directly quenching the energy in triplet chlorophyll and singlet oxygen (Young and Frank, 1996). In carotenoid-deficient plants, photodamage of plastids results in an albino phenotype (Vecchia et al., 2001).

The *immutans* (*im*) mutant of *Arabidopsis* is defective in carotenoid biosynthesis (Wetzel et al., 1994). Unlike most carotenoid biosynthetic mutants, *im* is variegated and able to survive to produce seeds. The green sectors of *im* have normal levels of chlorophyll and carotenoid pigments, while the white sectors accumulate phytoene, a non-photoprotective, colorless carotenoid precursor (Wetzel et al., 1994). The growth of *im* is very sensitive to light: it forms predominantly green sectors under low light conditions, while white sector
formation is enhanced under high light intensities. The fact that phytoene accumulates in the mutant indicates that \textit{im} is defective in the phytoene desaturase (PDS) step of carotenoid biosynthesis. PDS converts phytoene into \( \zeta \)-carotene (Cunningham and Gantt, 1998), and biochemical and genetic analyses have demonstrated that PDS activity requires PQ as an electron acceptor (Norris et al., 1995). The well-known herbicide, norflurazon (NF), is a competitive inhibitor of PQ for the PDS active site (Breitenbach et al., 2001). The cloning of \textit{IM} revealed that the IM protein is a distant homologue of mitochondrial alternative oxidase (AOX), which is a ubiquinol (UQH\(_2\)) oxidase (Wu et al., 1999; Carol et al., 1999). By analogy to AOX, it was early suggested that IM might serve as a plastoquinol (PQH\(_2\)) oxidase, with PQ serving as the electron acceptor of the PDS redox reaction. Consistent with this idea, IM has PQH\(_2\) oxidase activity \textit{in vitro} and \textit{in vivo} (Josse et al., 2000; Joet et al., 2002).

A model has been proposed to explain the \textit{im} variegation mechanism (Wu et al., 1999). In this model, IM function is essential during the early stages of chloroplast development when the photosynthetic electron transfer chain is not yet fully-formed. In the absence of electron acceptors downstream of PQ, a lack of IM would result in an accumulation of PQH\(_2\). PDS is not able to convert phytoene into \( \zeta \)-carotene without PQ, and thus photoprotective, colored carotenoid biosynthesis would be blocked at the PDS step. Consequently, developing plastids would become photooxidized. However, once chloroplasts are formed and the photosynthetic electron chain is functional, IM seems to be dispensable, because a functional photosynthetic electron chain can replace IM’s role in keeping the balance of the redox state of the PQ pool.
One of the most interesting aspects of *im* is that the cells of the plant have a uniform genetic composition, but the leaf cells have dramatically different phenotypes: white or green. Cells of *im* plants can also be heteroplastidic for defective and normal plastids (Wetzel et al., 1994). The mechanism of variegation in mutants with a uniform genetic background is not well-characterized (Rodermel, 2002). In this chapter of the thesis, I launched a proteomics analysis of the white sectors and green sectors of *im* to better understand the mechanism of *im* variegation. I was especially interested to define how nuclear and plastid genes respond to defective, white plastids caused by a mutation at the PDS step of carotenogenesis. NF treatment also causes plant photooxidation by inhibiting PDS activity, and NF-treated plants have frequently been used as models to study the influence of photooxidation on the regulation of nuclear gene expression (retrograde, chloroplast-to-nucleus signaling) (Strand et al., 2001; Rodermel., 2001). Thus, I incorporated NF-treated Arabidopsis into this research to better understand the effects of photooxidization on nuclear-plastid interactions. One question was whether leaves from NF-treated plants (carotenoid deficiency caused by an exogenous agent at the PDS step) have similar proteomic profiles to white sectors from *im* plants (carotenoid deficiency at the PDS step induced by an endogenous gene mutation).

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Seeds from wild type Arabidopsis (Columbia ecotype) and the *spotty* allele of *immutans* were germinated and grown at 22°C under continuous 15 μmolm\(^{-2}\)s\(^{-1}\) light, initially for 5 days (to allow for the formation of green sectors in *im* plants), then at 100 μmolm\(^{-2}\)s\(^{-1}\) continuous light until the samples were collected. For NF-treated plants, wild type
Arabidopsis were grown for three weeks, as described above, then watered with 5µM norflurazon every day for 1 week; newly emergent leaves were white. The white and green sectors were dissected from NF-treated plants and from im plants at the pre-bolting stage. Four different leaf samples were collected from randomly selected plants -- wild type (WT), white tissues from NF-treated plants (NF), green tissues from im plants (imG), and white tissues from im plants (imW). The tissues were ground in liquid nitrogen and stored at -80°C until further analysis.

**Sample preparation and protein determination**

Leaf samples (1 g) were ground into power in liquid nitrogen, then homogenized in 5 ml of 10% TCA (in cold acetone). Proteins were precipitated at -20°C for 1 hour, then washed three times in ice-cold acetone and air-dried. The protein pellet was solubilized at 25°C for 2 hours by adding 1 ml re-hydration buffer (6M urea, 2M thiolurea, 2 mM TBP, 0.125 M Tris-HCl, 1% IPG buffer), then centrifuged at 13,000rpm for 10 min. The supernatant was collected for 2-D gel analysis. The concentration of protein in the sample was measured using the Bio-Rad protocol with bovine serum albumin as a standard.

**2-Dimensional gel electrophoresis**

200 µg protein in a final volume of 250 µl was loaded onto 13 cm, pH 4-7 Immobilon DryStrips (Amersham Pharmacia Biotech, Uppsala, Sweden). After rehydration of strips for 2 hrs at 20°C, isoelectric focusing (IEF) electrophoresis was performed using the IPGphor focusing system (Amersham Pharmacia Biotech, Uppsala, Sweden) at 20°C with a maximum current of 50mA, using the following program: 20 V for 10 hrs, 100 V for 2 hrs, 500 V for 2
hrs, 1,000 V for 2 hrs, 2,500 V for 2 hrs, and finally 8,000 V until the total volt-hours reached at least 80,000.

After electrophoresis, the IPG strips were removed and equilibrated in an SDS-containing buffer (50mM Tris-HCl, 6M urea, 3% SDS 20% glycerol, 0.125% TBP, pH 8.0) for 30 min. The strips were then placed on top of 12.5% SDS-PAGE gels and sealed with 0.5% agarose in SDS running buffer. The SDS-PAGE gels were run with cooling water for 6 hrs using a Bio-Rad apparatus. After electrophoresis, a colloidal Coomassie blue staining method was used to visualize the protein spots.

**Analysis of 2-D gels**

Gel analysis and comparison were performed with PDQuest software using a protocol developed previously (Lonosky et al., 2004). In brief, 3 gel images for each tissue type were obtained using a GS-800 calibration densitometer (Bio-Rad, Hercules, CA); all protein gels were sized and orientated in the same way. Original gel images were transferred into Gaussian modeling images, on which all analysis was carried out. Spots with quality values greater than 30 were selected for further analysis; there were roughly 500 “high quality” spots generated for each gel. Four match sets were created -- WT vs imG, WT vs NF, WT vs imW, NF vs imW -- and each match set contained 6 gels (3 replicates from each different sample). Gels in each match set were aligned based on select “landmark” spots present at the same position on each of the gels. After alignment analysis, statistical analyses, quality analyses and quantitative analyses were conducted with relative density as the normalization method. A 3-fold change and significance level of p<0.05 were used as criteria to identify the differentially-regulated protein spots in a match set.
Protein identification by MALDI-TOF mass spectra and database searching

Differentially-regulated protein spots were manually cut out of the gels and placed into a 50% methanol solution. Each gel piece was washed at least 3 times with 2.5 mM Tris-HCl, 50% acetonitrile (pH 8.5) to remove SDS and the Coomassie blue dye. Gel pieces were dried in a speed vacuum until there was no color left in the gel. Sequencing-grade, modified trypsin was added to the dried gel pieces and in gel digestion was performed overnight at 37°C. Peptides from trypsin digestion were eluted from the gel pieces with elution buffer (50% acetonitrile, 0.5% trifluoroacetic acid) and applied to obtain a spectrum using a Voyager-DE Pro MALTI-TOF mass spectrometer (Perspective Biosystem).

The resulting peptide fingerprints were calibrated internally with 5 peptides from trypsin autodigestion using DataExplorer software (Applied Biosystems, Foster City, CA). The calibrated peptide fingerprints were then applied to search against the NCBI protein database to obtain protein identification with the error setting as 25 ppm using the MS-Fit web-based program (UCSF).

RESULTS

Proteomic profiles of im green tissues

Under the growth condition applied in this research, im plants have a variegation phenotype of white sectors and green sectors, while the NF-treated wild type plants display an albino phenotype in the young leaf tissues (Figure 1). It has been hypothesized that IM is dispensable once green sectors are formed, because the photosynthetic electron chain is able to replace IM's role to maintain the redox state of the PQ pool; therefore, the green sectors of
im plants are phenotypically normal (Wetzel et al., 1994; Wu et al., 1999). To test whether wild type (WT) and the green sectors of im (imG) are biochemically the same, I compared their proteome profiles using 2-D gel electrophoresis. Figure 2 and Table 1 show that, indeed, the wild type and imG profiles are strikingly similar. Among the approximately 500 spots analyzed, only five displayed a 3-fold or greater statistically-significant difference (p<0.05); all five were up-regulated. MALDI-TOF analysis revealed that two of these spots are glutathione S-transferase 1 (GST-1), and that the other 3 spots are glutathione S-transferase 2 (GST-2). If the criteria are decreased to a 2-fold difference, 8 additional spots are up-regulated in imG versus wild type. Seven of these spots correspond to 3 proteins: cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cytosolic glutamine synthetase (GS), and an endomembrane-associated protein. The last spot could not be identified. No protein was found to be significantly down-regulated in imG either with a 2-fold or 3-fold cutoff.

Proteomic profiles of im white tissues.

White tissues of im plants are the result of photooxidation due to a lack of carotenoids; plastids in the white sectors of im (imW) are depleted of carotenoids and chlorophylls and have vacuolated structures (Wetzel et al., 1994). Figure 3 shows that the proteomic profile of imW leaf tissues is remarkably different from that of WT: 131 (45 + 86) of the 500 total analyzed spots differed in intensity by 3-fold or more between the two tissue types (Tables 2 and 3).

Table 2 and Figure 3A show that 45 of the 130 spots are down-regulated in imW. 40 of these were positively identified, and they represent 15 different proteins, all with well-
defined functions. All of the down-regulated proteins are located in plastids and are components of photosynthesis or tightly involved with photosynthesis. These include components of the light reactions (PSI type III CAB, PSII type I CAB, PSII OEC 23, PSII OEC 33, PSII HCF 136, the Rieske Fe-S protein, and the ATPase α and β subunits) and the dark reactions (Rubisco and carbonic anhydrase). Also down-regulated in \( \text{imW} \) is Fe-SOD, which is in charge of scavenging \( \text{O}_2^- \), a toxic byproduct derived by electron leak from the photosynthetic electron transfer chain (Mckersie et al., 2000).

86 protein spots were up-regulated at least 3-fold in \( \text{imW} \) (Table 3 and Figure 3B). 8 of these spots could not be identified by MALDI-TOF, while the other 78 were found to represent 46 different proteins. 20 of the 46 were found to be chloroplast-localized, while 8 proteins were localized in mitochondria and the other 18 proteins in the cytosol.

Of the 20 chloroplast proteins, five are chaperones and three are involved in stress defense: a Cu, Zn-SOD and 2 peroxiredoxins (Alscher et al., 2002; Dietz, 2003). Peroxiredoxins are thioredoxin-dependent peroxidases, which are able to detoxify various peroxide substrates (Dietz, 2003). Interestingly, chloroplast Fe-SOD is decreased while Cu, Zn-SOD is up-regulated in \( \text{imW} \). Other up-regulated chloroplast proteins include two that are involved in lipid metabolism and four that are involved in RNA binding or protein synthesis. Other functions represented by single proteins include signal transduction, transport, phosphate metabolism and plastid division. Of the 18 up-regulated cytosolic proteins, four are involved in stress defense (including GST-1, GST-2, and GST-6); two are involved in cellular transport; four play a role in RNA binding, protein synthesis or degradation; and 8 represent other biological processes. Of the 8 up-regulated mitochondrial proteins, five are
involved in respiration, one is involved in RNA-binding, and two function in stress response (Mn-SOD and a GST).

**The proteomic profiles of NF-treated white tissues**

Norflurazone (NF) is a widely-used herbicide. It causes a blockage in the PDS step of carotenogenesis, which leads to an accumulation of phytoene and a consequent lack of colored carotenoid production (Breitenbach et al., 2001). It is commonly thought that NF results in a massive destruction of plastid proteins (Mayfield et al., 1986; Vecchia et al., 2001). To better understand the effect of NF on gene expression and to compare the effect of PDS disruption caused by NF treatment versus a defective *IM*, we undertook a proteomic analysis of NF-treated plants (NF). Figure 4 shows that the proteomic profile of NF is significantly different from that of WT, but that it shares a striking similarity with that of *imW* (compare Figure 4B with Figure 3B). Tables 4 and 5 reveal that 129 spots differ in intensity by 3-fold or more with a significant difference (*p*<0.05) in NF versus WT gels, with 43 spots down-regulated and 86 spots up-regulated.

Of the 43 down-regulated spots in NF-treated (versus WT) plants, four spots could not be identified, and the other 39 represent 17 different proteins. These 17 proteins are all localized in chloroplasts, and 15 of the 17 proteins are the same 15 that were down-regulated in the *im* white tissues. The other 2 proteins are Rubisco activase, and chloroplast glutamine synthetase. Further analysis showed that both of these proteins are down-regulated at least 2-fold in *imW* compared to WT.

Of the 86 up-regulated spots in *imW* versus WT, 6 could not be identified and the other 80 spots represent 45 different proteins. 13 of these are from chloroplasts, 8 are from...
mitochondria and the other 24 are located in the cytosol. Of the 13 chloroplast proteins, 5 are chaperones; two are involved in stress defense; three are involved in RNA-binding and protein synthesis; one functions in lipid metabolism and one plays a role in plastid division (FtsZ). All of the chloroplast proteins were also up-regulated in \textit{imW}.

Of the 24 up-regulated cytosolic proteins, 7 are involved in stress defense, three in RNA binding, three in protein synthesis and turnover, three in glucosinolate metabolism, and 8 in other processes. The majority of these proteins are also up-regulated in \textit{imW}. Of the 8 up-regulated mitochondrial proteins, 5 proteins are involved in respiration and are the same respiratory proteins that were up-regulated in the \textit{imW} tissues. One protein is involved in RNA-binding; and two proteins function in stress defense: Mn superoxide dismutase (Alscher et al., 2002) and cysteine synthetase (Berkwitz et al., 2002). Cysteine is a precursor of glutathione and it is generally thought high cysteine levels lead to high levels of GSH (Youssefian et al., 2001; Sirko et al., 2004).

\textbf{Comparison of proteomic profiles of NF samples and imW tissues}

The down-regulated proteins in NF-treated tissues and \textit{imW} are almost the same (Table 2 and Table 4). In a similar manner, the profiles of up-regulated proteins are very similar but with some differences (Table 3 and Table 5). Tables 3 and 5 show that compared to WT, there are 13 proteins up-regulated in NF but not in \textit{imW}, while another 13 proteins are up-regulated in \textit{imW} but not in NF. However, comparisons of Tables 3 and 5 are misleading, because this is not a direct comparison between NF and \textit{imW}. In other words, any of the 13 proteins up-regulated in one tissue could be also up-regulated in the other tissue compared to WT, but just not as high as 3-fold. For example, a protein is up-regulated to 3-
fold in \textit{imW}, and 2.5-fold in NF; this protein would be shown in Table 3 not in Table 5.

When comparisons were made directly between NF (Figure 4B) and \textit{imW} (Figure 3B), only 9 spots were found to be 3-fold different, and these 9 spots represent 5 different proteins (Table 6). Of these 5 proteins, only one protein has 3-fold or higher intensity in \textit{imW} than in NF, and it is a vacuolar ATPase \varepsilon subunit. The other 4 proteins (8 spots) displayed 3-fold or more intensity in NF than in \textit{imW}. These include a mitochondrial glycine-rich RNA binding protein (GRR protein), cytosolic glyceraldehyde-3-P dehydrogenase (GAPDH), cytosolic dehydroascorbate reductase (DHAR), and a jacalin lectin family protein.

\section*{DISCUSSION}

\textit{The \textit{im} green tissues are similar to wild type tissues.}

Previous biochemical analyses have shown that the green sectors of \textit{im} plants have similar levels of chlorophylls and carotenoids compared to wild type plants, and ultrastructural observations have revealed that chloroplasts in the \textit{im} green sectors display the same size, number, and shape as wild type plants (Weztel et al., 1994; Aluru et al., 2001). Also consistent with the idea that the green sectors of \textit{im} plants are phenotypically normal, Figure 2 and Table 1 showed that the proteomic profiles of WT and \textit{imG} are strikingly similar. Only two proteins (GST-1 and GST-2) are up-regulated by 3-fold or more in \textit{imG} (Table 1). Even when the cut-off value is decreased to a 2-fold change, only three more proteins are differentially-expressed in \textit{imG} (an undefined endomembrane protein, glutamine synthase and GAPDH). Such a small proteome difference between WT and \textit{imG} indicates that the \textit{imG} tissues are nearly normal, and supports the hypothesis that IM is not essential.
for plant cell development and growth after cells have escaped photodamage during early chloroplast and leaf development.

Although the imG and WT proteomes are very similar, it is interesting that GST-1 and GST-2 are significantly up-regulated in imG. GST-1 and GST-2 are the two most abundant proteins of the GST family, and they detoxify xenobiotics and organic peroxides, via glutathione (GSH) conjugation (Edwards et al., 2000; Dixon et al., 2002). GSTs are often induced under conditions provoking oxidative damage, such as heavy metals, pathogen attack, wounding, ethylene and ozone (Marrs, 1996; Lederer, and Boger, 2003). Previous work has demonstrated that the IM protein plays a role in the protection of the photosynthetic electron chain from over-reduction and the formation of ROS (Baerr et al., 2005; Rizhsky et al., 2002). The up-regulation of GST proteins is thus consistent with the idea that they are induced to cope with stress in the absence of IM.

Another difference in imG that might account for the up-regulation of the five proteins is a higher source-sink ratio in imG versus WT leaf tissues. The green sectors of im are surrounded by white sectors, and thus the green tissues must feed the white ones. In accord with the notion that the imG tissues have a higher source capacity than normal, it has been reported that there is a higher photosynthetic rate in imG than in WT leaf tissues (Aluru et al., 2001). The increase of GADPH might suggest that there are also higher glycolytic and TCA Cycle activities in imG. Arabidopsis cytosolic glycolytic enzymes, such as GADPH, are found in mitochondrial intermembrane spaces, and it has been suggested that glycolytic activities are tightly associated with mitochondrial TCA activities (Giege et al., 2003).

Interactions between the nucleus and plastids involve anterograde traffic, in which nuclear gene products control plastid gene expression; and retrograde traffic, in which
nuclear gene expression is regulated by signals from plastids (Rodermel, 2001). In imG, it is therefore possible that the up-regulation of the five nuclear genes for plastid proteins seen in Table 1 could be caused by signals from chloroplasts. Some plastid signals have been characterized recently (Rodermel, 2001; Surpin et al., 2002; Pfannschmidt, 2003), and these include products of photosynthesis, carotenoids, porphyrins, ROS, and the redox state of the plastoquinone (PQ) pool. In imG, carotenoids and chlorophylls are normal, so it is possible that ROS or the redox state of the PQ pool could serve as plastid signals to regulate nuclear gene transcription.

Plastids from imW and NF are photooxidized but still alive

Compared to WT, the down-regulated proteins in imW and NF are strikingly similar. All are tightly related to photosynthesis. It is obvious that photosynthetic capacity is destroyed by photooxidation due to a lack of carotenoids. This finding is consistent with previous studies showing that nuclear genes for photosynthetic proteins are generally transcriptionally repressed when the chloroplast is photooxidized (Mayfield et al., 1986; Oelmuller, 1989; Tonkyn et al., 1992; Vecchia et al., 2001; Castro et al., 2005). I did not find a down-regulation of any proteins from the cytosol or other cell fractions. This is consistent with previous reports that damage caused by photooxidization is limited to the plastid compartment of the cell (Oelmuller, 1989).

Clearly, the plastids in imW and NF tissues are depleted of pigments and photosynthetic ability, but they are still alive and a number of chloroplast proteins are up-regulated in them. These include five chaperones: Rubisco binding proteins (α subunit and β subunit), heat shock proteins (HSP) 70-1 and 70-2, and chloroplast chaperonin 21. Because
Rubisco was dramatically down-regulated in imW and NF-treated tissues, an up-regulation of the Rubisco binding proteins might indicate that plant cells try to keep Rubisco in the right conformation, but the photooxidative pressure is too great. The same may be the case for HSP70-1, HSP70-1, and chaperonin 21, which have been reported to be involved in the protein import machinery and in maintaining protein confirmation (Zhang and Glaser 2002).

Three of the up-regulated proteins were Cu, Zn-SOD, peroxiredoxin, and 2-cys peroxiredoxin. All of these are directly involved in ROS scavenging (Dietz, 2003). Very interestingly, chloroplast Fe-SOD was down-regulated in imW, while chloroplast Cu, Zn-SOD was up-regulated. The difference in expression of Cu, Zn-SOD and Fe-SOD has been reported before (Kurepa et al., 1997), and it might suggest that chloroplast Cu, Zn-SOD and Fe-SOD play different roles under stress conditions.

A number of proteins involved in RNA-binding and protein synthesis were also up-regulated in the imW and NF-treated tissues. Unlike nuclear gene expression, which is mainly regulated at the transcriptional level, chloroplast gene expression is primarily regulated at the post-transcriptional level, and chloroplast RNA binding proteins are fundamental factors in chloroplast RNA metabolism (Nakamura et al., 2004). The up-regulation of chloroplast RNA binding proteins suggests that plastid gene expression is altered to survive photooxidization.

The up-regulation of three major groups of chloroplast proteins (chaperones, stress defense proteins, and RNA binding proteins) could be a general response to stress. A similar observation has been made in Arabidopsis under high light stress (Phee et al., 2005). Consistent with this idea is my finding of the up-regulation of an ABC transporter that is
involved in protein degradation (Xu and Moller, 2004). This protein might help plastids turn over their proteins, since there are a lot of proteins destroyed by photodamage.

I also found that FtsZ1, a protein involved in plastid division (Osteryoung et al., 1998; Strepp et al 1998), is up-regulated in imW and NF. Both reduced and elevated levels of FtsZ1 suppress chloroplast division (Stokes et al., 2000; Kiessling et al., 2000). The high level of FtsZ1 in imW and NF suggests there is an inhibition of plastid division in white sectors. This would be an efficient way to save energy. Examination of tissue sections supports this conclusion (data not shown). Up-regulation of FtsZ1 proteins may be a common mechanism to suppress plastid division if plastids are not able to function normally.

After photooxidation, plastids lose their photosynthetic ability and seemingly become useless. The fact that defective plastids are still alive raises a question: why do plant cells keep their defective plastids rather than degrade them? One potential answer to this question comes from the observation of the interchangeability of different type of plastids. In cotyledons of young seeds before maturation, defective plastids could be converted into proplastids during seed dehydration; normal chloroplasts develop from proplastids during the germination process (Fu and Rodermel, unpublished data). Thus, defective plastids can serve as the source of future generations of viable plastids. This might be why they are preserved.

**Mitochondrial components are up-regulated by defective plastids**

It is generally believed that photodamage caused by carotenoid deficiency is exclusively limited to plastids, and even plastid envelopes are not significantly impaired (Oelmuller, 1989; Tirlapur et al., 1999). My proteomic data generally agree with this idea. Yet, previous studies on how defective plastids affect gene expression have focused on genes
related to photosynthesis (Surpin et al., 2002; Pfannschmidt, 2003; Gray et al., 2002). How defective plastids affect expression of genes not directly involved in photosynthesis has not been extensively examined and is poorly understood.

The cross-talk between plastids and mitochondria is a very attractive research topic to plant scientists. We already know that defective mitochondria can cause abnormal plastid development in variegation mutants, such as iojap and ncs (Han et al., 1992; Newton and Coe, 1986; Marienfeld and Newton, 1994; Hedtke et al, 1999). How do plastids affect mitochondria in plant cells? My proteomic data did not find any down-regulated mitochondrial proteins in the imW and NF-treated tissues, suggesting that the defective plastids in these tissues do not cause defective mitochondria. However, photooxidized plastids, though they are still alive, can not provide energy to the cell. The only energy source (other than glycolysis) is mitochondria. For this reason, it is not surprising that mitochondrial components are up-regulated in both NF-treated and imW tissues.

In addition to the up-regulation of respiratory components, I found an up-regulation of mitochondrial proteins involved in ROS defense. This can be explained if there is an elevated respiratory activity in the imW and NF-treated tissues, and if this results in more ROS production. In this case, the mitochondrial ROS defense system might be up-regulated to ensure normal mitochondrial activity.

**Plant cells alter various biological systems to adapt to defective plastids**

Both in NF-treated and imW tissues, a number of cytosolic proteins were up-regulated compared to WT. An up-regulation of stress defense proteins in the cytosol suggests that carotenoid deficiency not only causes stress in plastids, but also in the cytosol.
Up-regulation of proteins involved in protein synthesis and turnover indicates that cells have to adjust their patterns of protein accumulation and synthesis. Since glycolysis is tightly related to mitochondrial activity (Giege et al., 2003), it is not surprising to find that enzymes in glycolysis were up-regulated because there is a higher mitochondrial activity in the white tissues. Similar patterns of up-regulation of proteins involved in stress, protein synthesis and turnover proteins, and glycolysis were found when grapevine tissues were subjected to the herbicide flumioxazin, an inhibitor of chlorophyll biosynthesis (Castro et al., 2005).

Up-regulation of proteins involved in nitrogen metabolism is a common response of plants to stress, and it is thought that such proteins function in the remobilization and storage of amino acids resulting from massive degradation of abundant proteins (Mira et al., 2002; Liu et al., 2005; Teixeira et al., 2005). Consistent with this idea, two proteins involved in nitrogen metabolism were up-regulated in white tissues of im. The glucosinolate-myrosinase system provides an effective defense system against herbivores and pathogens; and the glucosinolate gene is usually induced by pathogen attack and herbivory, which is mediated by jasmonates (Kliebenstein et al., 2005; Wittstock and Halkier 2002). The up-regulation of glucosinolate metabolism enzymes suggests that the plastid signaling system shares some common components with the response system to herbivores and pathogens.

In addition to the aforementioned genes, a cytosolic annexin was up-regulated in both NF-treated and imW tissues. Annexin is a Ca$^{2+}$-dependent membrane binding protein that is thought to play an important role in Ca$^{2+}$-signaling in stress defenses (Lee et al., 2004). Again, this is consistent with the idea that defective plastids cause stress in the cytosol. Caffeoyl-CoA-3-O-methyltransferase (CCoAOMT) is an enzyme involved in lignin biosynthesis; and enzymes related to lignin biosynthesis are induced by various stress.
conditions, such as UV light, plant pathogens, wounding, and drought stress (Vincent et al., 2005). The up-regulation of CCoAOMT in both NF and imW suggests this protein may also play a role in response to stress.

**NF treatment has slightly different impact on gene expression compared to im mutation.**

NF treatment has almost the same effect on chloroplasts and mitochondria as the im mutation. The same group of proteins was down-regulated by both treatments, and the same group of proteins was up-regulated in chloroplasts and mitochondria. The most variability between the two treatments was seen for up-regulated cytosolic proteins: NF treatment led to up-regulation of 24 cytosolic proteins, while imW led to up-regulation of 18 cytosolic proteins. When imW 2-D gels were directly compared to NF gels, 9 spots (5 proteins) were significantly different between the two (Table 6). Only one of these proteins (a vacuolar ATPase ε subunit) had a higher amount in imW than in NF-treated tissues. The plant vacuolar H+-ATPase is essential in maintaining ionic and metabolic gradients across endomembranes, in activating transport processes and in vesicle dynamics (Jansinki et al., 2003; Gaxiola et al., 2002). These proteins could function in the transport of toxic byproducts caused by ROS into vacuole. The other 4 proteins had a higher amount in NF-treated than in imW tissues: a mitochondrial GRR protein, a cytosolic GADPH, DHAR, and a jasmonate-inducible lectin protein. The mitochondrial GRR protein is believed to play an important role in regulating mitochondrial gene expression at the posttranscriptional level (Vermel et al., 2002). GADPH is an enzyme involved in glycolysis and, as discussed above, is tightly associated with mitochondrial activity (Giege et al., 2003). DHAR is a ROS stress defense protein in charge of regeneration of ascorbate from dehydroascorbate; it is also involved in
the ROS defense system (Mittler, 2002). The jasmonate-inducible lectin protein, which is a
myrosinase binding protein, is involved in the glucosinolate-myrosinase defense system of
the Brassica family (Wittstock and Halkier, 2002; Kliebenstein 2005). The difference
between NF and imW indicates that NF is a more severe stress to plants.

The difference between NF and imW tissues may also reflect the fact that NF is a
xenobiotic to plant cells. The effect of NF on gene expression is not identical to the effect on
gene expression of defective plastids, even though NF leads to the formation of defective
plastids. For example, NF may regulate nuclear gene expression directly rather than via
defective plastids. To date, most studies on the regulation of gene expression by plastid
signaling have been carried out using defective plastids following NF treatment. This could
be risky when global gene expression is investigated because changes in gene expression
may directly be due to NF itself, rather than to signals from the defective plastids themselves.

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FIGURE LEGENDS

Figure 1. Plant image of wild type, spotty allele of im, and NF-treated wild type plants. All plants were photographed at four weeks after germination. Plant growth and NF treatment are as discussed in the “Materials and Methods”.

Figure 2. 2-D gels of leaf proteomes from WT and imG.
A. 2-D gel from WT; B. 2-D gel from imG. 200 μg of total leaf protein was loaded on each gel. The staining of the gel and analysis of the protein spots is described in “Materials and Methods”. The numbered spots correspond to those in Table 1. Spots 7290, 8203, 8204, 8115 and 8108 have a 3-fold or greater intensity than the corresponding spots on the WT gel; the other marked spots have a 2-fold intensity difference.

Figure 3. 2-D gels of leaf proteomes from WT and imW.
A. 2-D gel from WT; B. 2-D gel from imW. The 45 spots that are numbered on the WT gel are down-regulated in imW (versus WT) with a three-fold or greater intensity difference. The 85 spots that are numbered on the imW gel are up-regulated in imW (versus WT) with a three-fold or greater intensity difference.

Figure 4. 2-D gels of leaf proteomes from WT and NF-treated Arabidopsis.
A. 2-D gel from WT; B. 2-D gel from NF white tissue. The 43 spots that are numbered on the WT gel are down-regulated in NF-treated (versus WT) with a three-fold or greater intensity difference; and the 86 spots that are numbered on the NF gel are up-regulated in NF-treated (versus WT) with a three-fold or greater intensity difference.
FIGURE 2

SDS-PAGE MW (kD)

SDS-PAGE MW (kD)

pH

4

7
Table 1 Identification of protein differentially regulated in imG

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These protein spots correspond to the marked spots on Figure 2B. In this table and the following tables, the spot # was assigned by the PDQuest software when comparison analysis was applied to the gels. Acc # is from the NCBI nonredundant protein database. Protein location was predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP). Possible function was obtained according to previous publications. Cyt, Cytosol.

Table 2 Identification of proteins down-regulated in imW

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Table 3 Identification of proteins up-regulated in imW

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<td>mit</td>
<td>RNA-binding</td>
</tr>
</tbody>
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These protein spots are the same as labeled on Figure 4B.
<table>
<thead>
<tr>
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<th>Acc#</th>
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<tr>
<td>3008</td>
<td>15236359</td>
<td>GR RNA BP</td>
<td>mit</td>
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</tr>
<tr>
<td>3518, 3519, 4516, 5518</td>
<td>30684083</td>
<td>jacalin lectin family</td>
<td>cyt</td>
<td>glucosinolate metabolism</td>
</tr>
<tr>
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<td>15222848</td>
<td>GAPDH</td>
<td>cyt</td>
<td>glycolysis</td>
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<tr>
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<td>15223576</td>
<td>DHAR</td>
<td>cyt</td>
<td>stress defense</td>
</tr>
<tr>
<td>8201*</td>
<td>15237054</td>
<td>vacuolar ATPase ε</td>
<td>cyt</td>
<td>membrane transport</td>
</tr>
</tbody>
</table>

These protein spots showed a 3-fold change when NF gels were compared with imW gels. Spot 8201 (labeled with *) has 3-fold or greater intensity in imW gels than in NF gels. The other spots have a 3-fold or greater intensity in NF gels compared to imW gels.
CHAPTER 5. FUNCTIONAL SUBSTITUTION OF A CHLOROPLAST PROTEIN
WITH A MITOCHONDRIAL PROTEIN: AOX RESCUES THE IMUTANS DEFECT CAUSED BY A LOSS OF PTOX

A paper to be submitted to The Plant Physiolgy
Aigen Fu, Fei Yu, and Steven Rodermel

ABSTRACT

Arabidopsis *immutans* mutants are variegated with phenotypically normal green sectors and carotenoid-deficient white sectors. *IM* encodes a plastid terminal oxidase (PTOX) that bears similarity to the mitochondrial alternative oxidase (AOX), and both proteins are diiron carboxylate quinol oxidases (DOX). AOX functions as a terminal electron acceptor in the alternative respiratory pathway, and PTOX functions as a terminal oxidase that drives carotenoid biosynthesis during the early stages of chloroplast development. In the absence of PTOX, plastids are photooxidized due to a lack of carotenoid accumulation. PTOX and AOX have a similar active site (DOX Domain) structure, although plastoquinol is the substrate of PTOX and ubiquinol is the substrate of AOX. Other then the DOX Domain, the structures of AOX and PTOX differ: PTOX has an Exon 8-specific Domain and AOX has a specific dimerization domain (D-Domain). Both of these domains are required for activity in the respective proteins. Here we present data demonstrating that AOX can functionally replace the role of PTOX in chloroplasts. This was accomplished by showing that re-targeting of AOX to the chloroplast can complement the defect in *im* plastids, giving rise to non-variegated plants. We also found that when the D-Domain is deleted from AOX, the
truncated protein is better able to complement PTOX function, while addition of the PTOX-specific IM exon-8 sequence to AOX totally compromises function. To our knowledge, this is the first report of functional substitution of a chloroplast protein with a mitochondrial protein.

INTRODUCTION

Variegation mutants have played a significant role in the history of genetics and led to the discovery of non-Mendelian inheritance in the early 1900’s (Kirk and Tilney-Basset 1967, Tilney-Basset 1975). Cells in the green sectors of variegated plants have morphologically normal chloroplasts, whereas cells in the white sectors contain abnormal plastids that lack pigments and organized lamellar structures. Unlike albino plants, variegation mutants can survive and reproduce, and thus they provide an excellent system to study interactions between the nuclear and organelar (chloroplast and mitochondrial) genetic compartments. However, a very limited number of variegations mutants have been characterized at the molecular level (Rodermel, 2001; Sakamoto, 2003).

One of the oldest variegation mutants of Arabidopsis is immutans (im), first described by Rédei, Röbbelen and co-workers nearly 40 years ago (Redei, 1963; Redei, 1967 Röbbelen, 1968). Sectoring in im is light-sensitive, with high light promoting the formation of white sectors. Cells in the white sectors of im accumulate phytoene, a noncolored C_{40} carotenoid intermediate (Weztel et al., 1994). All of the steps of carotenoid biosynthesis occur in plastids by nuclear gene products that are imported into the organelle post-translationally (Hirschberg, 2001), and thus the accumulation of phytoene suggested that im is blocked in the activity of phytoene desaturase (PDS), which converts phytoene into $\zeta$-
carotene (Cunningham and Gantt, 1998). Colored carotenoids protect plant cells from photooxidation by quenching triplet chlorophyll and singlet oxygen (Havaux, 1998; Tracewell, 2001), and it was hypothesized that the white sectors of im arise as a consequence of photooxidation due to a lack of carotenoid accumulation early in leaf development when chloroplasts are developing from proplastids in the leaf primordium (Wu et al., 1999; Carol et al., 1999). According to this scenario, developing plastids in the green sectors, despite having a mutant genotype, are able to compensate in some manner for the im defect.

IM has been cloned and codes for a chloroplast protein that is distantly-related to mitochondrial alternative oxidase (AOX) (Wu et al., 1999; Carol et al., 1999). AOX is a ubiquinol oxidase in mitochondrial inner membranes that functions as the terminal oxidase of the alternative electron transfer pathway (Vanlerberge and McIntosh, 1997). It is found in all plants, and in some fungi and protozoa, and it is thought to provide an overflow mechanism for carbon metabolism. Under stress conditions, AOX is also thought to minimize the production of reactive oxygen species (ROS) from electron transport (Bartoli et al., 2005; Umbach et al., 2005; Fiorani, et al, 2005). Because of its resemblance to AOX, IM was early hypothesized to be a plastoquinol oxidase involved in carotenoid biosynthesis, especially during the early stages of chloroplast development when the photosynthetic membrane is being assembled and the electron transport chain is not fully-functional (Wu et al., 1999). According to this hypothesis, electrons are transferred from phytoene to plastoquinone (PQ) (via PDS) and from plastoquinol (PQH₂) to molecular oxygen (via IM). Hence, in the absence of IM, an overreduced PQ pool would result in a blockage of PDS activity, since electrons can not be transferred to PQ. This would lead to carotenoid deficiency and the formation of white sectors (Wu et al., 1999). Consistent with this
hypothesis, IM has plastoquinol oxidase activity *in vitro* and *in vivo* (Josse et al., 2000; Carol and Kunz, 2001). In addition to its role in carotenogenesis, IM is thought to be a component in the arsenal of plant responses to oxidative stress, as well as the long sought-after terminal oxidase of the chlororespiratory electron transfer chain, which co-exists with the photosynthetic electron transfer chain on the thylakoid membrane (Carol and Kuntz, 2001). Hence, IM is also designated “PTOX”, for plastid terminal oxidase. We will use this designation in this report.

AOX and PTOX are members of the non-heme diiron carboxylate oxidase (DOX) family of proteins (Siedow and Umbach, 1995; Berthold et al., Finneg et al, 2003; McDonald et al., 2003; Atteia et al., 2004). By analogy to the crystal structure determinations of non-plant members of this protein class, it has been proposed that the diiron centers of AOX and PTOX (within the “DOX Domain”, Figure 1) are coordinated by four carboxylate and two histidine residues on a four helix bundle (also designated in Figure 1) (Siedow and Umbach, 1995; Andersson and Nordlund, 1999; Berthold et al, 2000). Consistent with this model are EPR spectroscopy studies showing that a coupled di-iron center is present in AOX (Berthold et al., 2002), and *in vitro* mutagenesis studies showing that some putative Fe ligands are essential for AOX activity when tested in procarytoic systems (Berthold et al., 2002; Albury et al., 1998; Albury et al., 2002; Ajayi et al., 2002; Chandhuri et al., 1998; Affourtit et al., 2002). Taking advantage of an *in vitro* assay for PTOX activity and the availability of null *im* alleles, we used PTOX as a model system to test the functional significance of the six putative Fe-ligands *in vitro* and *in planta* (Fu et al., 2005). These experiments showed that these sites do not tolerate change, even conservative ones. We also tested the functional significance of a 16 amino acid sequence that is present
in all plant IM sequences, but absent from AOX. This sequence corresponds precisely to Exon-8 of the genomic sequence ("Exon -8", Figure 1). This domain appears to be essential for PTOX activity and stability \textit{in vitro} and \textit{in planta}.

AOX forms inactive dimers that are held together by a disulfide bond. Treatment with \(\alpha\)-keto acids or pyruvate is associated with reduction of this bond, the formation of monomers, and enzyme activation. The Cys responsible for dimerization are located within the D-domain, which is thought to facilitate dimer formation (Umbach and Siedow, 2000). Although little is known about the regulation of PTOX activity, the conserved Cys and D-Domain are missing in PTOX, suggesting that PTOX has a fundamentally different mode of regulation. This might not be surprising since phylogenetic analyses have revealed that PTOX and AOX, though they share a common eubacterial ancestor, diverged prior to the endosymbiotic events that gave rise to mitochondria and chloroplasts (Finnegan et al, 2003; McDonald et al., 2003; Atteia et al., 2004). The process of endosymbiosis subsequently led to the transfer of the symbiotic AOX and POTX genes to the nucleus, where they were re-formatted for targeting back to the organelle.

Despite the differences in regulation, substrate-specificity, and phylogenetic distance, we were intrigued by the structural relatedness of PTOX and AOX and by the similarities in function. Therefore we wanted to test whether AOX could functionally complement the \textit{im} defect when retargeted and expressed in chloroplasts. In this report we show that, indeed, AOX can functionally substitute for PTOX when expressed in \textit{im} chloroplasts. This is the first example of a mitochondrial protein that has been re-targeted and shown to function in the chloroplast. We next took advantage of this finding to perform domain swapping
experiments to better understand the function of the AOX-specific D-Domain and the PTOX-specific Exon-8 domain.

MATERIALS AND METHODS

Plant growth conditions

Seeds from wild type Arabidopsis (Columbia ecotype), the spotty allele of im, and transgenic im plants were germinated and grown at 22°C under continuous illumination at 15 μmol m⁻² s⁻¹ for 5 days, to allow for the formation of green sectors. The plants were then transferred to 100 μmol m⁻² s⁻¹ continuous illumination.

Plasmid constructs and plant transformation

The Arabidopsis AOX1a cDNA containing the whole coding sequence was obtained by RT-PCR using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA) with primers: 5'-caaggatccatgatgataactcgcggtggag-3' and 5'-gttggatcctcaatgatacccaattggagc-3'. For expression in mitochondria, the AOX1a cDNA was cloned in the sense orientation behind the cauliflower mosaic virus 35S promoter into the BamHI site of binary vector pB003 (a generous gift from Min Li, Iowa State University); the resulting plasmid is termed as WT-AOX. The plasmid was sequenced to verify there were no PCR errors.

For chloroplast expression, a fragment from AOX1a that contained the coding sequence lacking the mitochondrial targeting peptide (MTP) was generated by PCR using the following two primers: 5'-caatggatccccgctagcacgatcac-3' and 5'-gttggatctcatacccaattggagc-3'. For expression in mitochondria, the AOX1a cDNA was cloned in the sense orientation behind the cauliflower mosaic virus 35S promoter into the BamHI site of binary vector pB003 (a generous gift from Min Li, Iowa State University); the resulting plasmid is termed as WT-AOX. The plasmid was sequenced to verify there were no PCR errors.
rbcS chloroplast targeting peptide (CTP) in the binary vector pCB302-1, which is designed for targeting proteins into chloroplasts (Xiang et al., 1999). The resulting plasmid is designated **C-LAOX** (long AOX). A cDNA lacking MTP and its immediately-flanking 40 amino acid D-Domain (Umbach and Siedow, 2000; Figure 1A) was obtained by PCR of the AOX1a cDNA using the following two primers: 5'-cgacggatccccgatctgaagaagcatcatgttcc-3' and 5'-gttggatcctcaatgatacccaattggagc-3'. The amplified sequence was cloned in the sense orientation behind the rbcS CTP of pCB302-1; the resulting plasmid is termed **C-SAOX** (short AOX). The AOX1a fragments in C-LAOX and C-SAOX were also cloned into pCB302-2 (designed for targeting proteins into mitochondria) in the sense orientation behind the β-ATPase MTP (Xiang et al., 1999); the resulting constructs are designated **M-LAOX** and **M-SAOX**, respectively. All plasmids were sequenced to verify that no errors were introduced by the PCR manipulations.

Compared to AOX (Figure 1A), a 16 amino acid sequence is present in PTOX that corresponds to exon 8 of the genomic sequence (Fu et al., 2005). This sequence was inserted into the corresponding position of either the LAOX or SAOX fragments using the ExSite™ PCR-based site directed mutagenesis kit (Stratagene, La Jolla, CA) by 4 sequential steps (each step adding 12 bp). The sequences were then cloned into pCB302-1 to generate **C-L+8** and **C-S+8**. All constructs are summarized in Figure 1B.

The constructs were transferred into *Agrobacteria tumefaciens* by electroporation, and the floral-dip method (Clough and Bent, 1998) was used to transform *im* plants (the spotty allele). Bar-positive, T1 transgenic plants were selected on soil by spraying seedlings with a 1:2000 dilution of commercially-available Finale (AgroEvo, Montvale, NJ), which contains 5.7% glufosinate ammonium. PCR and Southern Blotting methods were used to
verify that the plants were transformed. Phenotypic analyses were performed on T2 generation plants.

Isolation of chloroplast thylakoid membranes

Intact chloroplasts were isolated from Arabidopsis using established methods (Chen et al., 2000; Perry et al., 1991; Resink et al., 1998). In brief, leaf tissues from 3-4 week old rosette leaves were homogenized in a mortar and pestle in GR buffer: 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl2, 1 mM MnCl2, 0.5g/L BSA, 5 mM ascorbate and 50 mM HEPES-KOH (pH 8.0). The homogenate was filtered through four layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 2,600g for 5 min. The resulting crude chloroplast pellet was resuspended in GR buffer, then loaded onto a Percoll gradient. After centrifugation for 10 min at 10,500g, intact chloroplasts were removed from the gradient and washed twice in a buffer containing 0.33 M sorbitol and 50 mM HEPES-KOH (pH 8.0). To remove soluble proteins, the chloroplasts were washed with a buffer containing 20 mM MOPS and 50 mM EDTA (pH 7.0). The concentration of proteins in the pellet was measured using the Bio-Rad protocol (Hercules, CA) with BSA as a standard. Chlorophyll concentrations were measured as previously outlined (Perry et al., 1991).

Detection of RNA and protein

Procedures for total cell RNA isolation and Northern blotting have been described (Chen et al., 2000). The nitrocellulose filters were probed with a radiolabeled Arabidopsis AOX1a cDNA sequences.
For Western immunoblotting, protein samples were electrophoresed through 12.5% SDS polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. The membranes were probed with a polyclonal IM antibody at a dilution of 1:3000 (Fu et al., 2005) or with a monoclonal AOX antibody at a dilution of 1:200 (Elthon et al., 1989). The SuperSignal West Pico chemiluminescence kit (Pierce, Rockford, IL) was used for protein signal visualization.

RESULTS

Expression of AOX in chloroplasts rescues the variegation phenotype of im

To test whether AOX can functionally complement the im lesion, we cloned an AOX1a cDNA fragment that lacks its mitochondrial targeting peptide (i.e., the LAOX fragment, Figure 1A) behind the rbcS chloroplast targeting peptide (CTP) in the binary vector pCB302-1, which is designed for expression of nuclear genes for chloroplast proteins (Xiang et al., 1999). As a control, LAOX was cloned behind the β-ATPase mitochondrial targeting peptide (MTP) in pCB302-2, which is designed for expression of nuclear genes for mitochondrial proteins (Xiang et al., 1999). The resulting plasmids—C-LAOX and M-LAOX (Figure 1B)—were transformed into im plants and putative transformants were selected by their BAR-resistance.

Figure 2A shows representative T2 transgenic lines. The C-LAOX plants, while still variegated, are significantly greener than im, suggesting that AOX is able to complement the im defect, at least in part. Retransformation of these lines with a CTP + LAOX fragment in another binary vector pBI121 (Fu et al, 2005), which contains the 35S promoter and the NPTII gene as the selective marker, did not produce greener plants (data not shown).
However, the transformants became greener as they aged (compare 3 weeks versus 5 weeks growth, Figure 2F). This stands in contrast to *im*, whose degree of sectoring does not change during development. In contrast to the C-LAOX plants, Figure 2A shows that *im* plants transformed with mitochondrial-targeted AOX (M-LAOX) do not rescue *im*. This lack of complementation was observed in hundreds of transformants, as well as in transgenic *im* plants that overexpressed a full-length *AOXIα* (WT-AOX), i.e., a construct that contained its own versus the β-ATPase MTP (data not shown).

To confirm these findings we measured the levels of AOX mRNA by Northern analysis and the levels of IM and AOX proteins by Western immunoblot analysis using polyclonal antibodies to PTOX (Fu et al., 2005) and a monoclonal antibody to AOX (kindly provided by T. Elthon, University of Nebraska)(Elthon et al., 1988). Figure 2B shows, as expected, that only the WT plants have detectable levels of PTOX protein, consistent with the idea that *spotty* is a null *im* allele. By contrast, AOX mRNAs (Figure 2C) and proteins (Figure 2D) are plentiful in the transgenic versus *im* and WT plants. *AOXIα* mRNAs and proteins are barely detectable in whole leaf extracts from WT plants unless the blots are exposed for a long time. Figure 2E shows that AOX proteins are present in thylakoid membranes of C-LAOX plants, but not in the thylakoids of M-LAOX plants. Considered together, the data in Figure 2 indicate that LAOX proteins are targeted to the chloroplast, where they insert in thylakoids and complement the *im* defect, at least in part. This suggests that AOX can serve as a plastoquinol oxidase in chloroplast membranes.

**AOX without the D-domain complements the variegation phenotype of *im* more efficiently**
Although C-LAOX is able to complement the \textit{im} defect, at least in part, we reasoned that an AOX that bore greater structural similarity to PTOX might be better able to function in chloroplasts. To test this hypothesis we deleted the D-domain from C-LAOX to generate C-SAOX; the D-Domain is an \textasciitilde 40 amino acid sequence that is not present in PTOX and might function in AOX dimerization (Figure 1A) (Umbach and Siedow, 2000). As a control, we generated a mitochondrial-targeted version of AOX that lacks the D-Domain (M-SAOX) (Figure 1B).

Screening of hundreds of T2 \textit{im} transgenic lines carrying M-SAOX did not reveal any plants that differed from \textit{im}. However, 24 C-SAOX lines were identified that were significantly greener than \textit{im}, but to variable degrees. Figure 3A shows two representative lines with moderate-green (C-SAOX-1) and nearly all-green (C-SAOX-4) pigmentation. Southern blotting indicated that there is a single insertion of the foreign gene in the C-SAOX-1 line, and 3 copies in the C-SAOX-4 line (data not shown). Figures 3B and 3C reveal that gene dosage in these two lines is positively correlated SAOX mRNA and protein levels. In addition Figure 3D shows that the SAOX proteins are localized in thylakoids. The control M-SAOX plants also have high levels of SOAX mRNA and protein, but (as expected) the protein is not localized in the chloroplast. Again, endogenous \textit{AOX} \textit{a} mRNAs and proteins are difficult to detect in whole leaf extracts (see WT and \textit{im} lanes). To test the dosage hypothesis, we re-transformed C-SAOX-1 and C-SAOX-4 with a CTP + SAOX fragment in pBI121. Both sets of transformations resulted in greener plants (data not shown). This is in contrast to the C-LAOX results (above), where re-transformation had no affect on phenotype. However, foreign protein levels are \textasciitilde 100-fold higher in the transgenic C-LAOX \textit{im} plants than in the transgenic C-SAOX-4 plants (data not shown).
Figure 4 shows that the C-SAOX plants, like the C-LAOX plants, become more green as they age. In fact, newly emergent leaves from the C-SAOX-4 line are all-green. This is not due to enhanced accumulation of SAOX protein (Figure 4B). These data suggest that either SAOX becomes more active in the newly emergent leaves of older plants and/or that the capacity to green is influenced by developmental factors.

Do the AOX mutant proteins and IM form dimers?

In mitochondria, the AOX dimer is the inactive form of the enzyme, and it is converted into the active monomeric form by α-keto acids or NAPH (Umbach and Siedow, 1993; Umbach et al., 1994). Figure 5 shows the dimeric and monomeric forms of the enzyme on SDS gels either in the presence or absence of DTT. DTT reduces the disulfide bond that holds the AOX dimer together. The proteins were isolated from plants that overexpress the wild type AOXIa (WT-AOX, Figure 1). In contrast to the mitochondrial form of the enzyme, the chloroplast-targeted version of AOX (LAOX) only forms monomers in the thylakoid. This shows that the D-Domain is not sufficient for dimer formation, at least in chloroplasts. We do not understand the appearance of a band midway between the monomer and dimer forms of LAOX in the +DTT lane, but we suspect it might be a precursor.

The fact that PTOX lacks the D-Domain and conserved Cys residues has led to the suggestion that it is a monomer. SDS gels of IM are consistent with the idea that PTOX functions as a monomer, since no dimeric forms are seen on SDS gels, either under reducing or non-reducing conditions (Figure 5).
AOX with the IM Exon-8 sequence does not complement the variegation phenotype pf im

We have previously shown that a 16 amino acid domain near the C-terminus of PTOX is essential for PTOX activity and stability (Fu et al., 2005). This domain corresponds precisely to Exon 8 of the genomic sequence, and it is missing in AOX. Sequences flanking this domain are conserved and can be aligned in PTOX and AOX, allowing us to pinpoint the position of the "Exon-8 insertion" in AOX with respect to PTOX.

To test whether the Exon-8 domain influences AOX activity in the chloroplast, we inserted it in-frame by in vitro mutagenesis into C-SAOX and C-LAOX to form the C-S+8 and C-L+8 constructs, respectively (Figure 1B). Hundreds of T1 and T2 im transformants were screened, but none appeared appreciably greener than im (Figure 6A). This suggests that this domain does not influence AOX activity in the plastid.

To confirm this hypothesis we tested whether the constructs are expressed in im plants. Figure 6B reveals that the transgenic lines contain very high mRNA levels compared to wild type and im. As expected, the size of the mRNA in the C-S+8 line is somewhat smaller than in the C-L+8 line due to the deletion of the D-Domain. Figure 6C shows a complicated pattern of protein bands corresponding to AOX in the transgenic plants. Again, the endogenous protein is barley detectable (see the WT and im lanes). The predicted sizes of the mature C-L+8 and C-S+8 proteins are indicated, and while we do not understand the other bands, they might be precursor or degradation products. Nevertheless, we conclude that the foreign constructs are expressed in the transgenic plants and that they give rise to proteins of the correct size. However, these proteins do not appear to be able to complement the im defect.
**DISCUSSION**

*IM* is a single copy gene in the Arabidopsis genome, and AOX is the closest homolog of PTOX even though they share only ~26% amino acid sequence identity. In this report we showed that AOX is able to complement the plastid defect in Arabidopsis plants that lack PTOX, the IM gene product, via overexpression in thylakoid membranes. To our knowledge, this is the first example of substitution of a chloroplast function by a mitochondrial protein. Previous attempts to re-engineer the coding location of nuclear and organellar genes have been limited to chloroplast genes that have been translocated to the nucleus and re-targeted back to the plastid – e.g., *psbA* (Cheung et al., 1988) and *rbcL* (Kanevski and Maliga, 1994) – and nuclear genes that have been expressed in the chloroplast genome, e.g., *rbcS* (Whitney and Andrews, 2001; Dhingra et al., 2004).

Although AOX and PTOX share structural similarities (the DOX domain), the fact that AOX is able to function in the plastid is surprising given that AOX and PTOX differ with respect to the presence of other domains that are required for activity, viz., the D-Domain and the Exon 8 Domain. It is also surprising since the two proteins have different substrates. While substrate-specificities have not been rigorously tested, our *in planta* data clearly suggest that PQ serves as the AOX substrate in plastids, since thylakoids lack ubiquinol. This stands in contrast to PTOX, where *in vitro* analyses have demonstrated that PTOX does not show ubiquinol oxidase activity when expressed in *E. coli* (Josse et al., 2003). Based on this finding, we would predict that PTOX is not functional in mitochondria, but this will be difficult to test, given that the Arabidopsis *AOX* gene family consists of five members and no phenotypically distinguishable *aox* mutants are available.
Role of D-Domain and Exon 8 in plastid-expressed AOX

The D-Domain in AOX contains the regulatory Cys and is thought to be important for the formation of AOX dimers. Mitochondrial AOXs are usually present in their activated monomer state (Millenaar and Lambers, 2003; Umbach et al, 2005). It is intriguing that im plants that overexpress SAOX, which lacks the D-domain, are much greener than im plants that overexpress LAOX. Accompanying these changes, the protein levels in the SAOX transgenic plants are lower than in the LAOX transgenic plants. There are two possibilities: the LAOX pool could contain a high proportion of inactive dimers; or SAOX could function as a plastoquinol oxidase more efficiently than LAOX. Results from the nonreducing and reducing gel analyses revealed that LAOX forms predominantly monomers, ruling out the first possibility. Therefore, we conclude that in plastids the form of AOX that lacks the D-Domain is more active than the form with the D-Domain, i.e., in plastids the D-Domain functions to inhibit activity (a new role for this domain). This would be consistent with the idea that SAOX better resembles PTOX structurally. Our finding that PTOX does not form dimers is also consistent with the idea that the active form of PTOX is the monomer and that inactivation does not appear to occur via dimer formation. This conclusion is consistent with 2-D green gel analyses (data not shown) and the suggestion from the absence a D-Domain in PTOX (Fu et al., 2005).

Using the same reasoning, we might have anticipated that the addition of Exon-8 to AOX would make it resemble PTOX more closely and thus serve to foster AOX activity in chloroplasts. Contrary to our expectations, we found that addition of the Exon-8 sequence did not enhance AOX’s ability to substitute for PTOX in chloroplasts; instead it made AOX totally incompetent to function. The exon-8 sequence is located between helix 3 and helix 4.
of PTOX, and very close to the iron binding sites (Fu et al., 2005). The addition of exon-8 to this site in AOX might therefore disrupt the fine structure of the AOX reaction center in some manner that is not readily identifiable.

Model of variegation

One of the intriguing aspects of the im variegation is why green tissues arise against a uniformly mutant genotype. We have previously suggested that this is because one or more factors are able to compensate for a lack of PTOX, and that this compensation is important during early chloroplast biogenesis when the components of the thylakoid membranes are being synthesized and fully-functional electron transport chains are not yet assembled (Aluru et al., 2004). In this case, transient over-reduction of the PQ pool might occur. PTOX is expressed in developing thylakoids (Aluru et al., 2001), and we propose that at this time it acts as a "safety valve" (Niyogi, 1999), providing an alternate pathway of electron flow from over-reduced PQ to oxygen. This would prevent the formation of toxic ROS and photooxidation of the contents of the plastid. In plants lacking PTOX, we hypothesize that the photooxidative pressure is below a threshold of photoprotection in some plastids and they are able to turn green; in other plastids the photooxidative pressure is above the threshold and the plastids turn white. Therefore, we hypothesize that factors that are able to compensate for a lack of PTOX might be able to boost the photoprotective potential of plastids, allowing them to better cope with a given level of photooxidative pressure.

In this report we show that an exogenously-supplied terminal oxidase is able to fulfill this compensatory role. We also found that im transgenic plants with overexpressed AOX in their chloroplasts turned greener and greener, and finally gave rise to all-green new leaves.
and reproductive organs. However, AOX levels remained constant during plant development. So, it seems that PTOX activity is more important in the early stages than in the later stages of development, and that developmental factors are also able to compensate for a lack of PTOX. This is not a rare phenomenon in plants, and there are several reports that chloroplast mutations are most pronounced in early developmental stages but that plants manage to overcome their deficiency later in development. Arabidopsis thfl T-DNA insertion mutants are severely stunted with variegated leaves, and the THF1 protein is a chloroplast protein involved in thylakoid biogenesis. Transgenic plants with the antisense Thf1 gene are stunted with an altered thylakoid structure in young seedlings, but later on the phenotype is not as strong (Wang et al., 2004). Similarly, Toc33-deficient plants are pale in the early stage of development with abnormal chloroplast structure but appear normal in later stages (Jarvis et al., 1998). Similar findings have also been reported for an Arabidopsis mutant with a defect in the 54kD subunit of the chloroplast signal recognition particle (cpSRP54); the mutant plants have pale cotyledons and yellow first true leaves but turn normal as they age (Pilgrim et al., 1998). Thus, it seems that plants are able to compensate for a reduction in gene products that are important for normal chloroplast development by some unknown mechanism(s).

In addition to AOX and developmental factors, we have tried to rescue im by selecting some key enzymes in the stress defense system as our target genes, and have overexpressed these genes in im plants (Fu and Rodermel, unpublished data). These include a chloroplast copper, zinc superoxide dismutase (Cu,Zn-SOD), a chloroplast iron superoxidase (Fe-SOD), a chloroplast ascorbate peroxidase (APX), chloroplast cysteine synthetase (chlCS), cytosolic cysteine synthetase (cytCS), glutathione S-transferases (GST-1 and GST-2), cytosolic
glutamine synthetase (GS), and glyceraldehydes-3-P dehydrogenase (GAPDH). However, none of these transgenic plants was greener than *im*. We do not know why these enzymes failed to have an impact on photoprotection, but it is possible that they were not active during early chloroplast biogenesis; that they did not provide the correct kind of photoprotective mechanism; or that they were active but did not contribute enough to boost a larger percentage of plastids over the photoprotection threshold. The fact that AOX was able to accomplish this task suggests that mechanisms that serve to quench an over-reduced PQ pool might be crucial during the assembly of the photosynthetic apparatus. Perhaps other chloroplast oxidases, when overexpressed in *im*, might be important safety valves during this important developmental time.

Our current hypothesis thus emphasizes the notion of a threshold of photoprotection and that PTOX plays an important role in achieving this threshold during chloroplast development. It is unlikely it contributes substantially after the photosynthetic apparatus has been formed since flux through PTOX has been estimated at less than 2% of the total photosynthetic electron flow in mature leaves (Peltier and Cournac, 2002). Nevertheless, we propose that green and white sectors in developing and mature leaves are primarily a reflection of large-scale differences in restrictive and permissive conditions perceived by the developing leaf, e.g., regions of the meristem that are shaded might translate into green patches in the mature leaf, whereas regions that perceive high light translate into white patches.

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**FIGURE LEGENDS**

**Figure 1.** AOX plasmid constructs.
A. Structure of PTOX and AOX. Both proteins have an N-terminal organelle targeting sequence (TP, transit peptide). AOX has a dimerization domain (D-domain), whereas PTOX has an Exon-8 sequence. The DOX domain is shown in gray. Six "*" stand for six iron binding sites. WAOX, the whole AOX amino acid sequence; LAOX, the AOX sequence minus the TP; SAOX, the AOX minus the TP and the D-domain.

B. The sequences between the right border and left border of T-DNA of constructs used in this study. P35S, 35S promoter of cauliflower mosaic virus; Tnos, terminator of nos (nopaline synthetase) gene; Bar, gene for phosphinothricin acetyltransferase; CTP, chloroplast targeting sequence of rbsS (Rubisco small subunit); MTP, mitochondrial targeting sequence from tobacco β-ATPase subunit gene; WAOX, full-length AOXla coding sequence; LAOX, AOXla coding sequence after MTP sequence; SAOX, AOXla coding sequence after D-domain sequence; L+8, LAOX sequence with insertion of IM exon-8 sequence at corresponding position; S+8, SAOX sequence with insertion of IM exon-8 sequence at corresponding position.

Figure 2. Expression of LAOX in im plants

A. Representative wild type (WT), im, im transformed with C-LAOX, and im transformed with M-LAOX. All plants were maintained at 100 μmolm⁻²s⁻¹ continuous illumination after an initial 5 days at 15 μmolm⁻²s⁻¹. Pictures were taken 4 weeks after germination.

B. 100 mg FW of leaf tissues were ground in 500 μl SDS sample buffer and 20 μl protein extracts were separated by SDS-PAGE with DTT. After transfer to nitrocellulose membrane, the blot was probed with an antibody to IM.
C. Northern blot analyses were conducted using 2 μg of total RNAs from leaves of WT, im, im transformed with C-LAOX, and im transformed with M-LAOX. The nitrocellulose filters were probed with an AOX1a cDNA.

D. 100 mg FW of leaf tissues were ground in 500 μl SDS sample buffer, and protein extracts (20 μl) were separated by SDS-PAGE with DTT. After transfer to a nitrocellulose membrane, the blot was probed with a monoclonal antibody to AOX.

E. Western immunoblot analyses were conducted using thylakoid membranes (corresponding to 5 μg of chlorophyll) from Percoll-purified chloroplasts. The filters were probed with a monoclonal antibody to Arabidopsis AOX. The samples were the same as in Figure 2A.

F. Images of transgenic im plants that overexpress C-LAOX at 3 weeks, 4 weeks, and 5 weeks after germination.

**Figure 3. Overexpression of SAOX in chloroplasts.**

A. Representative plants of wild type (WT), im, im transformed with C-SAOX line 1 (C-SAOX1), C-SAOX transgenic line 4 (C-SAOX4), and im transformed with C-SAOX.

B. the figure legend is the same as Fig. 2B

C. the figure legend is the same as Fig. 2C

D. the figure legend is the same as Fig. 3E

**Figure 4. Development of C-SAOX plants**

A. Images of im plants, wild type plants, C-SAOX1 plants, and C-SAOX4 plants at 3 weeks, 4 weeks, and 5 weeks
B. AOX level in the expanding leaves from im plants at 4 weeks, wild type plants at 4 weeks, C-SAOX4 plants at 3 weeks, 4 weeks, and 5 weeks. The protein extract and western bolting are the same as Fig. 2C.

**Figure 5.** Mitochondrial WT-AOX, chloroplast C-LAOX, and PTOX analyzed by reducing (+DTT) or nonreducing (-DTT) SDS-PAGE. D, dimmer band; M, monomer band.

**Figure 6.** Overexpression of L+8, S+8 in chloroplasts of im plants

A. Representative plants of wild type (WT), im, im transformed with C-L+8, and im plants transformed with C-S+8.

B. the figure legend is the same as Fig. 2B

C. the figure legend is the same as Fig. 2C. The band with right size were labeled with “∗”. 
FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
Variegation mutants are excellent systems to study interactions between the nucleus and cytoplasm. The Arabidopsis mutant *immutans* is induced by a nuclear recessive gene and the variegation is sensitive to light and temperature (Wetzel et al., 1994). While the green sectors contain cells with normal chloroplasts, the white sectors are devoid of pigments and accumulate a non-photoprotective colorless carotenoid, phytoene. A blockage in phytoene desaturase caused deficiency in carotenoid biosynthesis, which leads to photooxidation and formation of white sectors. Cloning of the *IMMUTANS (IM)* gene has revealed that *IMMUTANS (IM)* is a homolog of alternative oxidase (AOX), a mitochondrial inner membrane protein (Wu et al., 1999; Carol et al., 1999). Current research results have suggested that IM is a plastid terminal oxidase (PTOX) essential for chloroplast biogenesis and playing important roles in photosynthesis protection (Aluru et al., 2006). To better understand variegation mechanism of *im*, this thesis focuses on characterizing the structure and function of IM protein at different levels.

Based on crystal structures of other structurally related protein, 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 (Andersson and Nordlund, 1999; Bertold et al., 2000). As mentioned in chapter two, the functional significance of these residues was tested by site-directed mutagenesis *in vitro* and *in planta*. The six putative Fe-binding sites do not tolerate change, even conservative ones. The significance of a conserved sequence in (or near) the PTOX active site, which corresponds to Exon 8 of genomic sequence, was also examined. *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. Chloroplast import assays revealed that mutant enzymes lacking Exon 8 have enhanced turnover. The Exon 8 domain is required not only for PTOX activity but also for its stability.

Besides the six iron-binding sites, there are other 14 amino acid residues are highly conservative between PTOX and AOX sequence. In chapter three, alanine scanning mutagenesis were conducted on the 14 other conserved sites using in vitro and in planta assay procedures. The significance of these 14 sites falls into three classes: i) A139, E171, N174, L179, P216, A230, D287 & R293 are dispensable for activity; ii) Y234 & D295 are essential for activity; and iii) L135, P142, H151, and Y212 are important but not essential for activity. These results are consistent with the proposed role of some of these residues in active site conformation and/or catalysis. Titration experiments showed that downregulation of PTOX to ~3% of wild type levels did not compromise plant growth, at last under ambient growth conditions. This suggests that PTOX is normally in excess, especially early in thylakoid membrane biogenesis.

In Chapter four, I applied a comparative proteomic approach to analyze green sectors and white sectors of im. A small proteomic difference was found between wild type and im green tissues; it suggested green sectors are phenotypically normal but under a slight stress condition. The nature of down regulated proteins in im white sectors suggested that photosynthetic capacity was destroyed in white sectors, but the photodamage was primarily limited inside plastids. The nature of up regulated chloroplast proteins suggested that the plastids were alive but with an altered protein composition. The proteomic analysis also suggested there is a higher mitochondrial respiratory activity in white sectors, and cells were induced to change various biological pathways to adapt to photodamage. Norflurazon, a
herbicide inhibiting carotenoid biosynthesis, has a similar effect on the gene expression as \textit{im} mutation, confirming that defects in \textit{im} white sectors were caused by carotenoid deficiency.

In chapter five, I presented data to demonstrate that if AOX was directed to express in chloroplasts, it can functionally replace PTOX role in chloroplasts. Further, I found AOX deleted of dimerization domain is more efficient to function as PTOX in chloroplasts, while addition of IM exon-8 sequence into AOX make AOX incompetent to function as PTOX in chloroplasts.

The variegation pattern of \textit{im} plants IM does not play a significant role in proplastid life or differentiated plastid life; however, IM plays an essential role in the early differentiation stage from proplastids to chloroplasts. A down regulated FtsZ protein level and morphological analysis suggested that the division of the defective plastids was suppressed in \textit{im} white sectors. Based on the typical features of carotenoid deficiency mutants (Robertson et al., 1978; Oelmuller, 1989) and a hypothesis proposed by Wu et al (1999), I further proposed a hypothesis to interpret the \textit{im} variegation mechanism. In this hypothesis, differentiating plastids develop into defective plastids due to photo damage under high light condition without carotenoid protection; while some differentiating plastids escape photodamage and develop into normal chloroplasts due to multiple shade effects. Once functional thylakoid membrane is formed, it can replace IM’s role to drive carotenoid biosynthesis. In a differentiating heteroplastidic cell, chloroplasts keep post-mitotic division, while division of defective plastids was suppressed. Consequently, a differentiating heteroplastidic cell develops into a mature homoplastidic cell. Therefore homoplastidic and heteroplastidic differentiating cells with normal chloroplasts lead to green sector formation, while homoplastidic cells with defective plastids lead to white sector formation.
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