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# REDUNDANCY OR FLEXIBILITY: MOLECULAR DIVERSITY OF THE ELECTRON TRANSFER COMPONENTS FOR P450 MONOOXYGENASES IN HIGHER PLANTS

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## 1. ABSTRACT

Specific metabolic roles of P450-dependent monooxygenase systems are determined by enzymatic properties and substrate specificity of P450s, the terminal enzymes of the electron transfer chain. On the other hand, molecular diversity has also been reported for NADPH-cytochrome P450 reductase, cytochrome $b_5$, and cytochrome $b_5$ reductase in plants. Several lines of evidence indicate that the electron transfer components for plant P450 reactions have specific physiological roles. In this review, we describe the current status of knowledge of the biochemistry, molecular biology, gene regulation, and molecular diversity of plant P450-related electron transfer components and summarize possible individual physiological roles of the diversified P450 electron transfer systems in plants.

## 2. INTRODUCTION

Cytochrome P450 (P450) is a generic name of a family of heme-thiolate proteins catalyzing mixed-function monooxygenase reactions involved in oxidative metabolisms of a diverse array of endogenous and exogenous lipophilic substrates. For the microsomal monooxygenase reactions, redox proteins including NADPH-cytochrome P450 reductase (P450-reductase) and cytochrome (Cyt) $b_5$ plus its NADH-dependent reductase are involved in the electron transfer to the P450s as the terminal enzymes. In the mitochondrial electron transfer system, P450s together with the mitochondrial type ferredoxin and the NADPH-dependent reductase play essential roles in the steroid biosynthesis in animals.

In plants, a number of P450 genes have evolved for the production of an innumerable number of secondary metabolites (1, 2). Some of those plant metabolites are synthesized to organize and integrate essential biological processes during normal growth, while many secondary metabolites accumulate as defense responses under biotic and/or abiotic stress stimuli. For example, plant P450s are known to be crucial to the biosynthesis and metabolism of fatty acids, phytosterols, phenylpropanoids, terpenoids, phytoalexins, glucosinolates, and plant growth regulators including gibberellins, brassinosteroids, abscisic acid, jasmonic acid, and indole acetic acids. Furthermore, oxidative detoxification of a number of herbicides in plant tissues is also achieved by the P450-dependent monooxygenase system. Thus, exploring enzymatic properties of plant P450s has attracted considerable interests in light of the fundamental importance not only for understanding plant biology but also for unlimited potentials in both crop improvement and industrial applications (2).

However, until the early 1990's, plant P450 studies had been hampered due to the difficulties in purifying P450 proteins. Only a few plant P450 proteins could be purified and characterized in the reconstitution systems (3-6). Owing to the rapid progress of molecular biology, a number of P450 genes from a wide variety of plant species have so far been isolated and characterized. In Arabidopsis, a dicot model species for genomic studies, the annotated P450 sequences amounted to as many as 270 including pseudogenes. Recent review issues (1, 2) have intensively discussed plant P450 genes focusing on biological roles of the plant P450 monooxygenase reactions so far clarified. Specifically, molecular and genetic

analyses of Arabidopsis mutants have been successful to identify novel P450 functions in plants. However, even in Arabidopsis, physiological functions of most P450s still remain unknown, and the current major challenges in plant P450 research include systematic elucidation of physiological substrates and metabolic roles of such "orphan" P450s.

The electron transfer components are essential for P450 monooxygenase reactions, and the molecular diversity has also been reported for P450 reductase, Cyt $b_5$, and Cyt $b_5$ reductase in plants. However, specific metabolic roles of plant P450 systems are determined by the catalytic properties of the terminal electron acceptor, P450s, and therefore, the existence of molecular diversity of the electron transfer components has not been a major concern for plant biologists. Nonetheless, several lines of evidence indicated specific metabolic roles of electron transfer components for plant P450 reactions (7-9). In this review, we describe the current status of knowledge of the biochemistry, molecular biology, gene regulation, and molecular diversity of such P450-related electron transfer components in plants and try to discuss possible individual physiological roles of diversified P450 electron transfer systems in plants.

## 3. NADPH-CYTOCHROME P450 REDUCTASE

### 3.1. Molecular Diversity

P450-reductase, a flavoprotein containing FAD and FMN, functions in plants transferring two electrons from NADPH to P450s on the endoplasmic reticulum (ER) membrane. However, in contrast to mammals and yeasts, it is likely that each plant species contains multiple isoforms of P450-reductase. For example, three isoforms of P450-reductase proteins were purified from the microsomal fraction of *Helianthus tuberosus* (10), and two P450-reductase proteins from *Petunia hybrida* flowers by Mentig *et al*. (11). Ponnamperuma and Croteau (5) have also detected two P450-reductase proteins from an extract of spearmint (*Mentha spicata*) glandular trichomes. These results suggested that each plant species might contain several NADPH-P450 reductase isoforms, however it was not clear whether these reductases were encoded by distinct genes or they were post-translationally modified proteins encoded by a single gene.

Molecular cloning studies substantiated the presence of P450-reductase isoforms in plants. Some initial studies identified a single gene in mung bean (12), periwinkle (13), and *Vicia sativa* (accession Z26252). Also in tobacco, only a single form of a full-length cDNA for NADPH-P450 reductase has been reported (14), whereas the amphidiploid tobacco genome could contain several NADPH-P450 reductase genes.

Benveniste's group has isolated two P450-reductase cDNAs from *H. tuberosus* (unpublished results; GenBank accession numbers Z26250 and Z26251, respectively) and confirmed their first report of multiple P450-reductases in this species (10). Two P450-reductase cDNA clones (*Petroselinum crispum*) have been obtained from parsley (15), and two cytochrome P450 reductase isoforms were identified in *Papaver somniferum* and *Eschscholzia californica* (California poppy) (16).

Two P450-reductase isoforms in Arabidopsis were characterized in more detail (8, 17). When recombinantly expressed, both P450-reducases in Arabidopsis were fully functional in transferring reducing equivalents to a plant P450, cinnamate 4-hydroxylase (CYP73B5), from Arabidopsis, and their biochemical and enzymatic properties were indistinguishable. Striking differences were found in the primary structures of the proteins. The N-terminal portions of the P450-reductase proteins have no similarity in either length or sequence, implicating distinct intracellular localization. No further report has been published with regard to the subcellular localization of the P450-reductase isoforms in Arabidopsis. This was also the case for the reductase isoforms in hybrid poplar (*Populus trichocarpa* x *Populus deltoides*). Ro *et al*. (18) isolated cDNAs for three P450-reductase genes from the hybrid poplar. Two isoforms (CPR2 and CPR3) were very similar (91% identical) at the amino acid level, while CPR1 exhibited a sequence identity of only about 72% compared with CPR2 and CPR3. When C-terminal green fluorescent protein (GFP) fusions to CPR1 and CPR2, of which N-terminal structures were significantly divergent, were expressed, both proteins localized to the ER in Arabidopsis. The differences in the N-terminal sequences of the reductases from the hybrid poplar were not involved in different subcellular localization.

### 3.2. Regulation and defense responses

P450-reductase isoforms could be involved in different physiological roles through differential regulation of gene expression. One of the mRNA species encoding P450-reductases in parsley (15) was specifically accumulated in response to biotic and abiotic stresses such as the fungal elicitor treatment and UV-irradiation. In the subtropical plant *Catharanthus roseus*, the levels of P450-reductase gene transcript were enhanced by fungal elicitor preparations that induce terpenoid indole alkaloids biosynthesis and P450 gene expression (19).

Mizutani and Ohta (8) reported a differential gene regulation of two P450-reductase isoforms (*AR1* and *AR2*) in Arabidopsis. RNA blot analysis showed that *AR1* was constitutively expressed throughout development, whereas *AR2* was strongly induced by wounding and light treatments. The *AR2* induction was coordinated with the induction of the genes encoding phenylalanine ammonia-lyase (*PAL1*) and cinnamate 4-hydroxylase (*CYP73A5*) in the general phenylpropanoid pathway, which is the primary metabolism activated in response to abiotic stresses such as wounding. Sequence analysis revealed that these genes shared potential sequence motifs for transcription-enhancing cis-regulatory elements involved in the plant responses to wounding and light signal. By using monoclonal antibodies raised against the recombinant P450-reductases (AR2), it was confirmed that the AR2 protein accumulated in the tissues of the wounding treatment

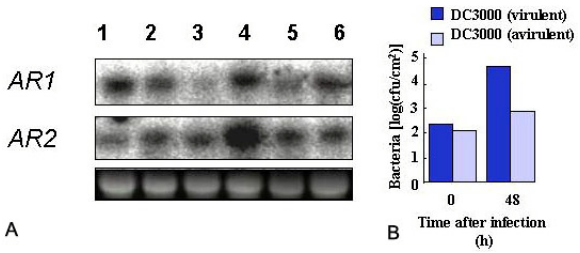


**Figure 1.** Accumulation of AR2 protein. For the wounding treatment, Arabidopsis leaves were cut using a razor blade and were incubated in potassium phosphate buffer (pH 6.5) in a phytobox (22°C) under continuous illumination. Protein blot analysis was performed using two different rabbit anti-AR1 antibodies (1 and 2) and two independent monoclonal antibodies (3 and 4) raised against recombinant AR2 protein (8). The specificity of the monoclonal antibodies was confirmed using the recombinant AR1 and AR2 proteins (8). Experiments 1 and 2 detected both AR1 and AR2 proteins, and experiments 3 and 4 demonstrated that AR2 protein specifically accumulated upon wounding.

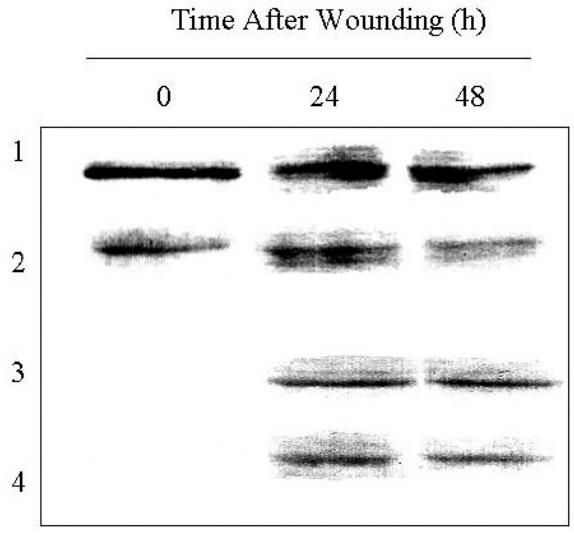


**Figure 2.** *AR1* and *AR2* gene induction in Arabidopsis leaves. Arabidopsis seedlings (ecotype Columbia, Col-0) were infected with the avirulent pathogen of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), which carries the plasmid pV288 containing the avirulence gene *avrRpt2* [*Pst(avr-Rpt2)*] (21) or the virulent *Pst* pathogen without the avirulence gene *avrRpt2*. RNA was prepared from local (infected) leaves and systemic (not infected) leaves of the same seedlings, and RNA-gel blot analysis was performed using gene specific probes (8). (A) Accumulation of *AR1* and *AR2* gene transcripts were analyzed after 48h of the infection with either the avirulent or virulent strains of *Pseudomonas syringae* pv. *tomato* DC3000. 1, systemic leaves of mock infected seeglings; 2, local leaves of mock infected seedlings; 3, avirulent strain (systemic); 4, avirulent strain (local); 5, virulent strain (systemic); 6, virulent strain (local). The amounts of RNA loaded were indicated at the bottom panel. (B) Bacterial growth in the infected seedlings. During 48h of the infection experiment, the growth of the avirulent strain [*Pst(avr-Rpt2)*] was inhibited (figure 1). The specific induction of *AR2* gene expression was also observed during the induction of disease resistance responses in Arabidopsis (figure 2).

Plants can turn on defense-signaling pathways through differential activation of distinct defense pathways depending on the type of pathogens encountered. A classic example is systemic acquired resistance (SAR) that is activated in many plant species including Arabidopsis and tobacco by pathogen infection. It establishes defense systems in the infected plants against subsequent attacks by a broad spectrum of plant pathogens in distant, uninfected plant parts (20).

A model experiment (figure 2) was done by challenging Arabidopsis (ecotype Columbia, Col-0) with the avirulent pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), which carries the plasmid pV288 containing the avirulence gene *avrRpt2* [*Pst(avr-Rpt2)*] (21). Upon infection with *Pst(avr-Rpt2),* Arabidopsis plants develop SAR, while no or little SAR-related responses was observed when infected with the virulent *Pst* pathogen without the avirulence gene *avrRpt2*. As shown in figure 2, the accumulation of *AR2* gene transcripts was specifically high in the local (infected) tissues compared with the systemic (uninfected) tissues, and the induction was much stronger in the leaves infected with the pathogen carrying the avirulence gene *avrRpt2* [*Pst(avr-Rpt2)*]. These results suggested that the *AR2* induction could occur as one of the plant defense responses during the SAR development.

In Arabidopsis, a P450 gene, CYP71B15, has been identified to be essential in the biosynthesis of camalexin, an indole derivative that is the only phytoalexin detected in this plant and is involved in resistance to a fungal pathogen (22). CYP72A1 from *Catharanthus roseus* (Madagascar periwinkle) has been shown to be the secologanine synthase in the biosynthesis of indole alkaloids (23). In the Arabidopsis genome, 9 CYP72A genes including one psedudogene form a gene cluster on chromosome 3. Maleck *et al*. (24) reported a genome-wide transcriptome profiling during the induction of SAR in Arabidopsis. Among the genes investigated, a P450 gene (At3g14690), perhaps a gene belonging to CYP72A subfamily, was induced 8-fold in systemic leaves upon infection with *Pst(avr-Rpt2)*. However, little information is available for physiological functions of CYP72 genes in Arabidopsis and their involvement in the development of SAR. CYP72As in Arabidopsis may be involved in the biosynthesis of defense-related compounds yet unknown.

### 3.3. Dual flavoproteins in plants

Most flavoproteins contain either FMN or FAD as their prosthetic groups. On the other hand, dual flavoproteins containing both cofactors have been identified in a wide range of organisms including microbes, plants, insects, and mammals. These enzymes include NADPH-P450 reductase, nitric oxide (NO) synthase, bifunctional P450 enzyme containing a P450 reductase domain, CYP102 (P450BM-3) enzyme, and methionine synthase reductase. In plants, mammalian types of NO synthases and CYP102-like proteins have not been identified.

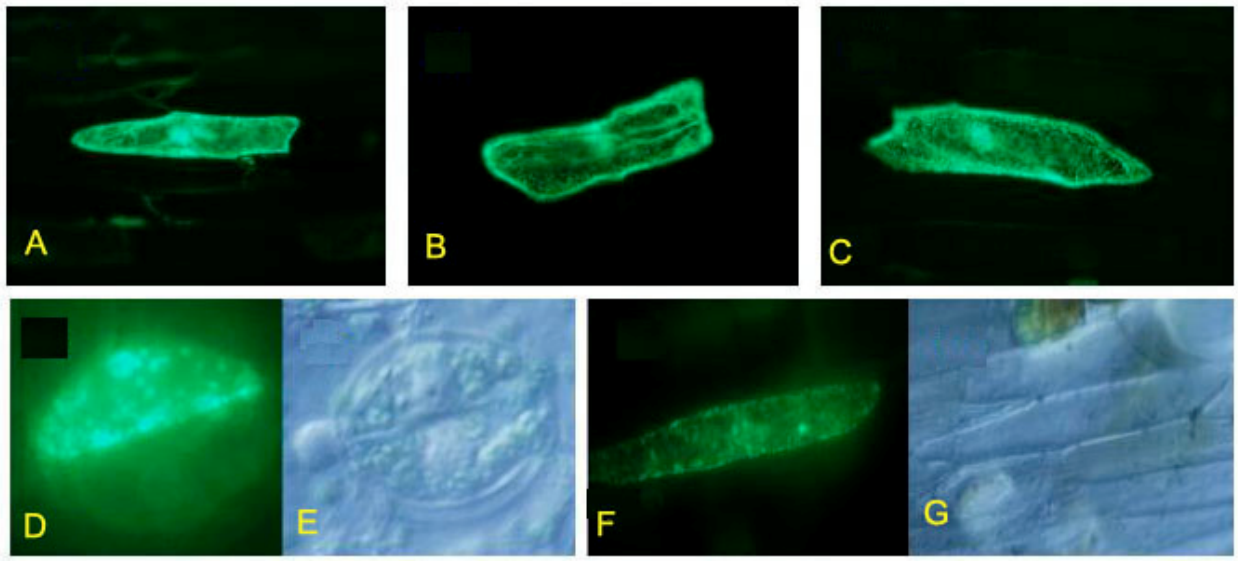


**Figure 3.** Phylogenetic tree for ferredoxin proteins. The clustalW program was used for the analysis of AtMFDX1 (AB07538), AtMFDX2 (AB081937), Arabidopsis ferredoxins (AtFDX-1, AAD15602; AtFDX-2, AAB65481; AtFDX-3, BAB09421; AtFDX-4, CAB10628; Q9SRR8, AAK62394), *A. vinelandii* Fdx (AAC24477), *B. taurus* ADX1 (AAA30358), *Chlamydomonas reinhardtii* FDX (AAC49171), *Drosophila melanogaster* Fdx (AAF50293), *Escherichia coli* FDX (NP_289082), *Homo sapiens* ADX(NP_004100), *Mesembryanthemum crystallinum* ferredoxin I precursor (AAB61593), *Oryza sativa* RFDX (BAA19865), *O. sativa* FDX1 (AAB65699), *Rattus norvegicus* ADX (P24483), *Spinacia oleracea* FDX1 (AAA34028), *Pseudomonas putida* PDX(BAA00414), *S. cerevisiae* Yah1p (NP_015071), and *Zea mays* FDX1(AAA33460). The GenBank/EMBL/DDBJ accession numbers for the proteins are indicated in the parentheses. AtMFDX1 (AB07538) and AtMFDX2 (AB081937) are shown in bold-type.

Recently, two NO-generating enzymes have been identified in tobacco (25) and Arabidopsis (26). These proteins in tobacco and Arabidopsis are homologous to the P protein of the glycine decarboxylase complex and a group of hypothetical bacterial proteins with GTP binding domains, respectively, but there is no sequence similarity to mammalian types of NO synthases.

When BLAST searches were performed using the amino acid sequences of P450-reductases, a putative gene (At3g02280) was identified to encode a P450-reductase-like protein in Arabidopsis. This putative NADPH-ferrihemoprotein reductase (At3g02280) exhibits a high sequence identity (41%) with human Nr1, a novel dual flavoprotein oxidoreductase of whose cellular function has not been elucidated (27). It has recently been reported that the *fre-1* gene, the *Caenorhabditis elegans* ortholog of human Nr1, is transcribed as a bicistronic pre-mRNA together with *dcs-1* encoding a Hint-related 4meGMP-directed hydrolase (28). However, no dcs-1-related sequence is detected in Arabidopsis. Another novel dual flavoprotein, methionine synthase reductase, found in mammals has been demonstrated to serve as a redox partner for methionine synthase (29). However, no sequence corresponding to the methionine synthase reductase protein has been detected in plants. It should be noted that plant methionine synthase catalyzes the methylation of homocysteine using the triglutamate form of methyltetrahydrofolate as the methyl donor and that both S-adenosylmethionine and cobalamine are not required for enzyme activity (30). In mammals, it is likely that the redox pathways for the methionine synthase activity are redundant. Thus, the pair of P450-reductase and Cyt $b_5$ was fully functional for the activity of methionine synthase *in vitro*, and the Nr1 protein was also sufficient to activate methionine synthase in the presence of Cyt $b_5$ (29). It remains to be clarified whether these reconstituted electron transfer activities were physiologically functional *in vivo*. Specifically, redox partner proteins of the putative NADPH-ferrihemoprotein reductase (At3g02280) in Arabidopsis may constitute a novel metabolic pathway in plants, and reverse genetic approaches could provide clues to explore new redox processes in plants. At least two independent lines with transfer-DNA (T-DNA) insertions tagging the gene of At3g02280 are available (The Arabidopsis Information Resource; http://www.arabidopsis.org/). The use of T-DNA from *Agrobacterium tumefaciens* as a mutagen is that the insertion of the DNA element into the Arabidopsis genome occurs at randomly selected locations, and the T-DNA saturation mutagenesis has been the powerful tool in the functional genomics (31, 32).

## 4. MITOCHONDRIAL TYPE FERREDIXINS AND FERREDOXIN REDUCTASE

### 4.1. Mitochondrial redox system

Adrenodoxin (ADX) is the [2Fe-2S]-type mitochondrial ferredoxin that mediates electron transfer from NADPH via adrenodoxin reductase (ADR) to the terminal electron acceptor, mitochondrial P450s (33). In vertebrates, the mitochondrial P450s catalyze the reactions of the cholesterol side chain cleavage and the steroid hydroxylation in the steroidogenic tissues (34). Yeast mitochondrial proteins, Yah1p (35) and Arh1p (36), correspond to ADX and ADR homologues, respectively.

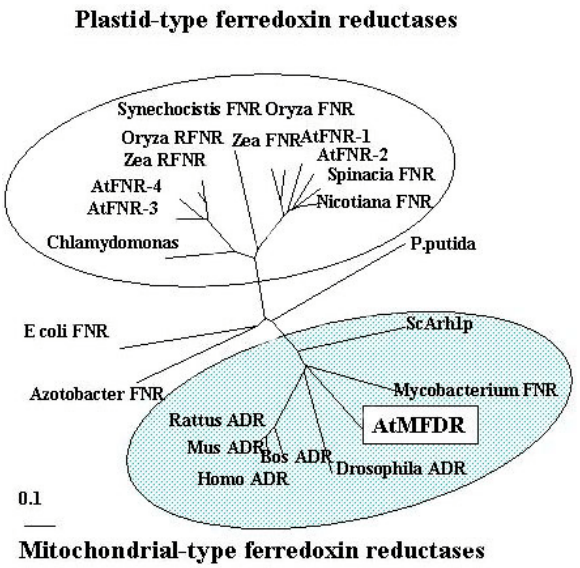


**Figure 4.** Phylogenetic tree for ferredoxin reductase proteins. The clustalW program was used for the analysis of AtMFDR protein sequence with the sequences of *A. thaliana* ferredoxin reductases (AtFNR1, AAF79911; AtFNR2, CAB52472; AtFNR3, CAB81081; AtFNR4, NP_174339), *A. vinelandii* FNR (AAA83029), *B. taurus* ADR1 (AAA30362), *C. reinhardtii* FNR (AAA79131), *D. melanogaster* ADR (AAD50819), *E. coli* FNR (CAA66094), *H. sapiens* ADR1 (AAB59497), *M. musculus* ADR (BAA08659), *M. tuberculosis* FNR (AAK47528), *N. tabacum* FNR (CAA74359), *O. sativa* FNR (BAA90642), *O. sativa* RFNR (BAA07479), *P. putida* PNR (BAA00413), *R. norvegicus* ADR (BAA23759), *S. oleracea* FNR (AAA34029), *Synechocystis* sp. PCC 6803 (NP_441779), *S. cerevisiae* Arh1p (AAC49500), *Z. mays* FNR (BAA88236), and *Z. mays* RFNR (AAB40034). The GenBank/EMBL/DDBJ accession numbers for the proteins are indicated in the parentheses. AtMFDR protein is indicated in bold-type

However, in yeast, no mitochondrial P450s exist, but Yah1p (37) and Arh1p (38) have been found as the essential proteins in the processes of Fe-S cluster maturation. Prokaryotic microorganisms also synthesize Fe-S cluster (39, 40) through the functions of proteins including a [2Fe-2S] ferredoxin, the Yah1p homologue encoded by the *isc* operon.

### 4.2. Mitochondrial electron transfer components in plants

Through BLAST searches with bovine ADX and ADR protein sequences at The Arabidopsis Information Resource (http://www.arabidopsis.org/), gene annotations are detected to be corresponding to the mitochondrial electron transfer components. Two gene annotations are thought to be encoding proteins (AtMFDX1 and AtMFDX2) highly similar to ADX (figure 3), and a putative gene is corresponding to a plant homologue of adrenodoxin reductase (AtMFDR) (figure 4). Takubo *et al*. (41) reported that these putative genes were actually transcribed to produce functionally active proteins for the electron transfer. Subcellular fractionation analyses suggested membrane association of AtMFDR protein, and protein-gel blot analyses and transient expression studies of GFP fusions indicated mitochondrial localization of AtMFDX1 and AtMFDR (figure 5). It should be noted that no GFP signals were seen from chloroplasts when the GFP fusions were expressed in tobacco epidermis (figure 5). RNA-gel blot analyses revealed that the accumulation levels of *AtMFDXs* and *AtMFDR* gene transcripts were specifically high in flowers (41).

### 4.3. Physiological roles

Ferredoxins and ferredoxin reductases are widely distributed in organisms across kingdoms and are involved in a variety of redox metabolic pathways (42). In photosynthetic organisms, these electron transfer components participate in the generation of NADPH during the linear photosynthetic electron transport. On the other hand, plant plastidal ferredoxin and ferredoxin reductase in non-photosynthetic tissues play crucial roles in nitrogen assimilation. In mammals, ADX and ADR constitute the mitochondrial electron transfer system for the P450-dependent steroid hydroxylation (34).

The subcellular localization study (figure 5) suggested that physiological partner proteins of AtMFDXs and the AtMFDR should exist in plant mitochondria. Considering the mammalian ADX and ADR functions, it is possible that AtMFDX and AtMFDR may be coupling to unidentified mitochondrial P450s in plants. Both AtMFDX and AtMFDR proteins contained the conserved amino acid residues (41) involved in the interaction with the mitochondrial P450s (33). In Arabidopsis, more than 270 P450 genes have been identified (The Arabidopsis Genome Initiative, 2000), and several of these P450 genes may encode mitochondrial P450 isoforms. If this is actually the case, the higher transcript levels of *AtMFDX* and *AtMFDR* genes in flowers (41) may suggest the presence of such P450s in flower buds or during flower development. It has been reported that steady state levels of some P450 gene transcripts including *CYP76C1* and *CYP91A2* were higher in flowers compared with other organs (43). Xu *et al*. (44) performed a microarray analysis to survey expression profiles of Arabidopsis P450 genes and identified that transcript levels of several P450s, such as *CYP710A4* and *CYP89A9*, were high in the reproductive organs. Additionally, some P450 genes such as *CYP71B22*, *CYP72A10*, *CYP86A4*, and *CYP96A9* were expressed at higher levels in flower (Arabidopsis MPSS database, http://mpss.udel.edu/at/java.html). However, the PSORT program did not give high probabilities for mitochondrial localization of any of these P450 proteins. It has been reported that somatic embryos of *Digitalis lanata* might contain the activity of mitochondrial cholesterol side chain cleavage (45). The reaction in mammals is catalyzed by a mitochondrial P450 (P45011A) (34).

### 4.4. Fe-S cluster maturation

Yeast mitochondrial proteins, Yah1p and Arh1p, represent the homologues of ADX and ADR, respectively. Interestingly, no mitochondrial P450s exist in yeast, while Yah1p (37) and Arh1p (38) participate in the Fe-S cluster maturation and mitochondrial iron homeostasis. In prokaryotic

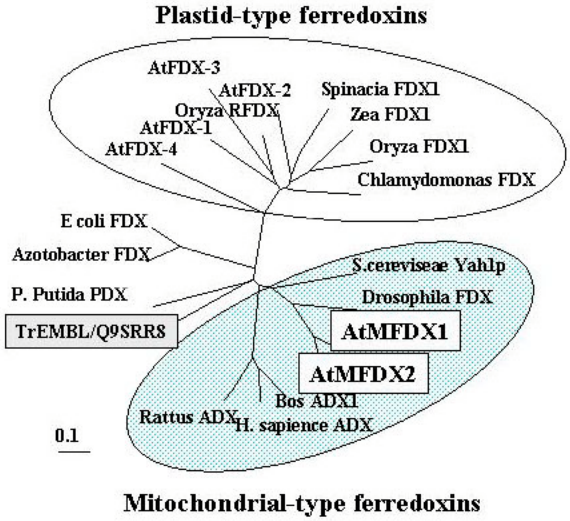


**Figure 5.** Transient expression of the AtMFDX1/S-GFP and the AtMFDR/S-GFP fusion proteins in onion epidermal cells and tobacco epidermal leaf cells. Expression of the fusion proteins was under control of the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator. The plasmids were constructed as described (41). For the transient expression, 25 µL of gold particles (1-µm diameter in 20% glycerol) were mixed with 5 µg of plasmid DNA, 50 µL of 2.5 M $CaCl_2$, and 25 µL of 0.1 M spermidine. Aliquots of gold were spotted on macrocarriers and used to transform tissues at 1100 psi using a PDS 1000HE biolistic device (Bio-Rad, CA, USA). (A), onion epidermal cells transformed with the control vector, sGFP(S65T); (B), AtMFDX1/S-GFP; (C), AtMFDR/S-GFP fusion proteins. The AtMFDX1/S-GFP and AtMFDR/S-GFP constructs were also used for transient expression in tobacco epiderimis. (D) AtMFDX1/S-GFP fluoresce was observed in a guard cell, (E) bright field image of the same cell of (D). (F) is the fluorescence image from a tobacco leaf epidermal cell expressing AtMFDR/S-GFP, and (G) is a bright field image from the same cell as (F). Fluorescence of GFP was visualized with an Olympus microscope IX71 (Olympus, Tokyo, Japan) and a filter set of U-MGFPHQ for GFP. Images were taken with the DP manager software (Olympus)

microorganisms (40), the assembly of Fe-S cluster is mediated by the functions of a series of proteins including a [2Fe-2S] ferredoxin, the Yah1p homologue encoded in the *isc* operon. Similar processes are also operating in the mitochondria of eukaryotic cells (37, 46), and the mitochondria of human cells contained an analogous system for Fe-S cluster maturation (47). Thus, these electron transfer components are presumably involved in Fe-S cluster biosynthetic processes in both prokaryotes and eukaryotic cells. Protein-gel blot analyses demonstrated that AtMFDR protein accumulated constitutively in leaves (41). This constitutive accumulation of AtMFDR protein may be attributable to the involvement in the Fe-S cluster biosynthesis as those reported for eukaryotic and prokaryotic microorganisms. It has recently been reported that *STARIK* gene in Arabidopsis encodes a half-type ABC transporter, which is a functional homologue of yeast Atm1p involved in the export of mature Fe-S cluster from mitochondria (48). The Arabidopsis genome contained putative genes encoding proteins homologous to those involved in the microbial Fe-S cluster maturation processes (40). For example, At5g65720 on chromosome 5 is encoding a NifS-like aminotransferase, and predicted genes (At4g22220, At4g04080, and At3g01020) probably encode NifU-like proteins. Therefore, it is possible that Fe-S cluster biosynthesis in plants may also follow a process closely resembled to those in other organisms, and AtMFDX and AtMFDR proteins may be involved in the process of cellular Fe-S cluster biosynthesis in Arabidopsis.

### 4.5. Mitochondrial proteome

Mitochondrial P450 proteins have not been identified by proteomic approaches (49, 50). Millar *et al*. (49) found an adrenodoxin/mitochondrial ferredoxin homologue (TrEMBL accession number Q9SRR8 encoded by At3g07480) in the Arabidopsis mitochondrial proteome. In the phylogenetic tree (figure 5), this ADX homologue (Q9SRR8 protein) was not in the mitochondrial ferredoxin group but very close to putidaredoxin, the electron transfer component involved in the P450cam reaction of *Pseudomonas putida*. The TargetP and the PSORT programs predicted the chloroplastic localization for the Q9SRR8 protein sequence. Sequence comparison indicated that the Q9SRR8 protein is missing one of the four Cys residues involved in the [2Fe-2S] cluster binding (33, 42) and the acidic amino acid residues involved in the interaction with ferredoxin reductase (33). These observations indicated that this ADX homologue could not function in the mitochondrial redox pathway.

Physiological redox partners of AtMFDX and AtMFDR remain to be elucidated. The amino acid residues for protein-protein interactions (33) are fully conserved in AtMFDX and AtMFDR proteins (41), and these electron transfer components in Arabidopsis could be sufficient for the mitochondrial P450 reactions. Generating transgenic plants with a mitochondrial type P450 from animals would offer an interesting option to verify whether plant mitochondria could serve as another site of P450 monooxygenase reaction.

## 5. CHLOROPLASTS

Allene oxide synthase (CYP74A) and hydroperoxide lyase (CYP74B) catalyze the reaction to form different classes of bioactive oxylipins from fatty acid hydroperoxide substrates within chloroplasts (51-53). The catalytic properties of these P450s do not involve the electron transfer from reductases and the activation of molecular oxygen. These P450s were localized to different envelope membranes of chloroplasts via distinct targeting pathways. Nonetheless, no electron transfer is required for the reactions by CYP74 subfamily enzymes.

Helliwell *et al*. (54) studied the subcellular localization of the enzymes of the early steps of gibberellin biosynthesis using fusions of green fluorescent protein. Gibberellin is synthesized from geranylgeranyl diphosphate

through several steps catalyzed by the terpene cyclase, ent-kaurenoic acid oxidase (CYP88A), and 2-oxoglutarate-dependent dioxygenase enzymes. Their results indicated that the leader sequences of the two CYP88A proteins from Arabidopsis were sufficient to direct GFP to the ER, while chloroplast import assays showed that the P450 protein was targeted to the outer surface of the chloroplast envelope. Thus, the CYP88A protein is most likely localized to the ER, and may be also associated with chloroplasts by keeping metabolic interactions with other plastid localized-enzymes, terpene cyclases, copalyl diphosphate synthase and ent-kaurene synthase, of the GA biosynthetic pathway. Using *in vitro* import assays with isolated pea chloroplasts, Watson *et al*. (55) reported that CYP86B1 of unknown function in Arabidopsis was also associated with the outer chloroplastic envelope membrane. Thus, CYP86B1 may also utilize substrates from metabolic pathways related to chloroplasts.

Ro *et al*. (18) reported that isoforms of the multiple P450-reductases from hybrid poplar were not localized to chloroplasts. It remains elusive that the chloroplastic redox pathways could support P450 reactions on the outer membrane or that an alternative P450 reduction system may be operative in this organelle. However, the reducing equivalent could be transferred from the ER-localized P450-reductase to the P450s associated with the chloroplastic outer surface. The intracellular membrane systems may interact each other in a dynamic way more than expected.

Lacour and Ohkawa (56) reported the yeast expression studies of fusion proteins of rat cytochrome P4501A1 with maize ferredoxin I (Fd) and pea ferredoxin NADP(+) reductase (FNR), the last electron transfer proteins of the photosynthetic channel in plant chloroplasts. The encoded fusion proteins P4501A1-Fd, P4501A1-FNR, P4501A1-Fd-FNR, and P4501A1-FNR-Fd were accumulated in yeast microsomes and were able to catalyze P450-monooxygenase activities towards 7-ethoxycoumarin and the herbicide chlortoluron. These results indicated that the chloroplastic electron transfer system could support P450 monooxygenase reactions *in vivo* when eukaryotic P450s are expressed in plant chloroplasts.

## 6. CYTOCHROME $B_5$ AND FUSION PROTEINS

The NADH-dependent electron transfer system comprising NADH-Cyt $b_5$ reductase and Cyt $b_5$ is also present in higher plants. NADH-Cyt $b_5$ reductase is the major electron-transfer component involved in lipid-modification reactions, and Cyt $b_5$ has been shown to function as an intermediate electron donor in the fatty acid desaturation of the microsomal membranes from developing safflower cotyledons (57, 58) and in the C5(6) desaturation of sterol precursors in maize as well (59). In the genome of Arabidopsis, six genes for Cyt $b_5$ proteins (At2g46650, At5g48810, At5g53560, At2g32720, At1g60660, At1g26340) are found (figure 6). The gene annotation of At1g60660 is predicted to code for a unique type of Cyt $b_5$ protein with a longer N-terminal extension but lacking the C-terminal domain conserved in the other Cyt $b_5$ proteins in Arabidopsis. Since the C-terminal amino acid residues of Cyt $b_5$ contain the information for the ER membrane targeting (60), this unique Cyt $b_5$ may not be localized to ER but to other subcellular organelles. Besides the gene encoding the ER-type NADH-Cyt $b_5$ reductase (At5g20080) (61), another putative gene for a mitochondrial-type Cyt $b_5$ reductase (At5g17770) is also present in the Arabidopsis genome.

Cyt $b_5$ is found not only as a single-domain protein but also in fusion forms. In Arabidopsis, for example, Cyt $b_5$ domain is found in nitrate reductase, delta-8 sphingolipid desaturase (*sld1*, At3g61580), and a putative fatty acid desaturase/Cyt $b_5$ fusion protein (At2g46210). Putative steroid binding proteins (At2g24940, At5g52240, At3g48890) also contain Cyt $b_5$-like domains. Another putative gene, At5g09680, is predicted to encode a polypeptide containing a Cyt $b_5$ domain at the C-terminal portion and the N-terminal domain of no significant homology with known functional categories.

In plants, it has been reported that Cyt $b_5$ reductase proteins are involved in NADH-dependent $Fe^{3+}$-chelate reduction (62). In maize roots, Cyt $b_5$ reductase was found to be targeted to the tonoplast, and no ER or mitochondrial localization could be detected (62). If this was actually the case, alternative ER-associated electron donors for the desaturases should be present. In mammals, Cyt $b_5$ reductase proteins are localized to multiple subcellular sites. Co-translational N-myristoylation is involved in the targeting to mitochondria and ER (63). On the other hand, two forms of Cyt $b_5$ reductase are produced from one gene: a myristylated membrane-bound enzyme expressed in all tissues, and a soluble, erythrocyte-specific isoform (64).

In higher plants, these electron transfer components are characterized by the gene multiplicity and duplication, though it is not known whether each isoform may participate in specific physiological roles. Utilizing Petunia plants, de Vetten *et al*. (9) demonstrated that a flower specific Cyt $b_5$ is involved in the regulation of the activity of a specific P450 catalyzing the 3', 5'-hydroxylation of dihydroflavonols, precursors of purple anthocyanins.

## 7. SUMMARY AND PERSPECTIVE

Metabolic channeling, metabolon, is the term to explain efficient metabolic activities conferred through substrate transfer from one enzyme to another in a certain metabolic pathway, with consequent reduction in metabolite pool sizes, reduction in lag times, increase in metabolic rates, protection of intermediates, and reduction of diffusive barriers (65-67). The metabolon is understood on a basis of multienzyme complexes catalyzing sequential reactions in a metabolic pathway, and is well described with several metabolic pathways including cyanogenic glucoside biosynthesis in sorghum (6), isoprenoid biosynthesis (68), phenylpropanoid and flavonoid pathways (69, 70).


**Figure 6.** Amino acid sequence alignment of the 6 Cyt $b_5$ proteins in Arabidopsis. The deduced amino acid sequences of At2g32720, At5g48810, At5g53560, At2g46650, At1g26340, and At1g60660 were analyzed using the clustalW program. Dashes indicate gaps inserted to allow optimal sequence alignment. Conserved amino acid residues were shaded and indicated by asterisks.

The genes encoding enzymes involved in a metabolon need to be under control of a coordinate transcriptional regulation to form the metabolic unit. Furthermore, for the specific metabolon organization, each enzyme also needs to have specific targeting information within the amino acid sequence or their secondary/tertiary structures. In other words, even if genes for metabolic units are coordinately regulated at the transcriptional level, a mechanism that organizes the metabolon is required to support the formation of specific metabolic units. Membrane systems or some other cytoskeletal structures may serve as the platform for the organization of unique metabolons (68). Winkel-Shirley (69) has proposed a metabolon of the phenylpropanoid pathway that contains P450s, although the model still lacks the role of electron donors for the pathway enzymes. The involvement of specific Cyt $b_5$ in the activity of dihydroflavonol 3', 5'-hydroxylase (9) suggested that electron transfer components could be also a member of metabolons.

Aiming at accumulation and production of plant metabolites, a number of approaches have been examined. Construction of fused-enzymes of P450 terminal enzymes with redox partner proteins is a promising alternative for integrating metabolic channels to create an efficient and desirable metabolic flow. Future challenges in plant metabolic engineering studies include the understanding of the molecular mechanism underlying integration of specific set of terminal enzyme P450s and the electron transfer components into the metabolic channeling with other pathway enzymes.

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**Abbreviations:** P450: cytochrome P450, Cyt: cytochrome, ER: endoplasmic reticulum, GFP: green fluorescence protein, SAR: systemic acquired resistance, Pst: *Pseudomonas syringae* pv. *tomato* DC3000, T-DNA: transfer-DNA, ADX: adrenodoxin, ADR: adrenodoxin reductase, Fd: ferredoxin, FNR: ferredoxin NADP(+) reductase

**Key Words:** Cytochrome, P450, Reductase, Ferredoxin, Cytochrome b5, Arabidopsis, Metabolic engineering, Review

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