Unique Features of Plant Mitochondrial Sulfhydryl Oxidase*

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The yeast and human mitochondrial sulfhydryl oxidases of the Erv1/Alr family have been shown to be essential for the biogenesis of mitochondria and the cytosolic iron-sulfur cluster assembly. In this study we identified a likely candidate for the first mitochondrial flavin-linked sulfhydryl oxidase of the Erv1-type from a photosynthetic organism. The central core of the plant enzyme (AtErv1) exhibits all of the characteristic features of the Erv1/Alr protein family, including a redox-active YPCXXC motif, noncovalently bound FAD, and sulfhydryl oxidase activity. Transient expression of fusion proteins of AtErv1 and the green fluorescence protein in plant protoplasts showed that the plant enzyme preferentially localizes to the mitochondria. Yet AtErv1 has several unique features, such as the presence of a CXXXXC motif in its carboxy-terminal domain and the absence of an amino-terminally localized cysteine pair common to yeast and human Erv1/Alr proteins. In addition, the dimerization of AtErv1 is not mediated by its amino terminus but by its unique CXXXXC motif. In vitro assays with purified protein and artificial substrates demonstrate a preference of AtErv1 for diethiols with a defined space between the thiol groups, suggesting a thioredoxin-like substrate.

Yeasts Erv1p (essential for respiration and vegetative growth) and the human homologue Alr (gusmeril of liver regeneration) were identified as the first mitochondrial sulfhydryl oxidases (1, 2). Their activity is essential for the survival of the cell, for the biogenesis of mitochondria, and for the supply of cytoplasmic proteins with mitochondrially assembled iron-sulfur clusters (3). Recently, these enzymes have been identified in a large number of different eukaryotic cell types and tissues (4).

As FAD-dependent sulfhydryl oxidases, these enzymes use molecular oxygen to form disulfide bonds coupled with the generation of hydrogen peroxide (5–7). The mitochondrial enzymes have a low enzymatic activity and seem to be adapted to the special requirements of this compartment. The catalytic core of yeast Erv1p comprises only about 100 amino acids and contains the FAD-binding site and the redox-active YPCXXC motif (1). This highly conserved ERV1 domain is the hallmark of the Erv1/Alr protein family (8, 9). During evolution, the fusion of the catalytic ERV1 domain with a thioredoxin domain generated a new facile catalyst for disulfide formation with dramatically enhanced enzymatic activity (6, 10, 11). This new protein family of FAD-dependent sulfhydryl oxidases performs diverse and important functions in higher eukaryotes (for a review see Ref. 9). The first complete sequence of such an ERV1/thioredoxin fusion protein was derived from a human protein that was differentially expressed as fibroblasts enter quiescence (6, 10). Based on this phenotype, these proteins were termed quiescin/sulfhydryl oxidases (QSOX).1 In these enzymes, the ERV1 domain contains the FAD-binding site and the redox-active primary CXXC motif. The first structural data for yeast Erv2p (12) and human AlrP (13) exhibit a unique four-helix bundle conformation for FAD binding. Recent publications demonstrate that interdomain redox communication between the primary redox-active CXXC motif and additional cysteine pairs is another characteristic feature of sulfhydryl oxidases (11, 12, 14). The adaptation of sulfhydryl oxidases to specific functions at different subcellular locations seems to correlate with the position and arrangement of these additional cysteine pairs (12, 14).

QSOX enzymes are not found in mitochondria but rather at many other subcellular locations and are also excreted from cells (9). QSOX enzymes have adapted to a broad spectrum of functions for cellular development and are thought to be involved in the formation of the extracellular matrix and in redox regulation of enzymatic activities (5, 10). The general importance of these enzymes is demonstrated by their presence in all multi-cellular organisms including plants. Recently, data base screening identified two genes for potential QSOX enzymes in Arabidopsis thaliana (9). In contrast, no mitochondrial sulfhydryl oxidase of the yeast ERV1-type has been described to date for any photosynthetic organism. This prompted us to investigate the model plant A. thaliana for the presence of such a mitochondrial sulfhydryl oxidase.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Escherichia coli strains used included DH5α for cloning experiments (15) and BL21 for expression of recombinant His-tagged proteins (Novagen). The yeast strains and their genetic markers are listed in Table I. The bacterial and yeast plasmids and vector constructs are listed in Table II.

PCR and DNA Cloning—The plant AtERV1 cDNA was amplified by standard protocols (21) using the a polymerase kit (Fermentas), a cDNA library from A. thaliana (Clontech library FL4000AB), and the primers listed in Table III. The restriction sites NdeI and XhoI were used for inserting PCR fragments into the hexahistidyl tag vector pET-24a (+) (Novagen). Primers with EcoRV sites allowed cloning of the PCR products, either with or without a His6 carboxyl-terminal extension, into yeast expression vectors. EcoRV/KpnI fragments were inserted into pBSC-preCytb2. After fusion with preCytb2, the open reading frame

1 The abbreviations used are: QSOX, quiescin sulfhydryl oxidase; At, A. thaliana; GFP, green fluorescent protein.
Reverse primer for 3
Reverse primer for deletion of last 15 amino acids
Reverse primer for deletion of last 10 amino acids

50 mM KH₂PO₄, 10 mM imidazole, pH 7.5) (Qiagen) (1, 22). The proteins
content, the 0.1-ml samples were diluted with 800 μl reagent) (23, 24) was added to a final concentration of
KH₂PO₄, 300 mM imidazole, pH 7.5. Purification to homogeneity was
ments were inserted into GFP vectors as listed in Tables II and III.
was excised as a BamHI/KpnI fragment and inserted into the compat-
carboxyl terminus of the proteins allowed rapid purification on nickel-
strain BL21 (Clontech). The presence of the hexahistidyl tag at the
PCR constructs encoding the full-length protein (22 kDa) and a carbox-

mM potassium phosphate buffer, pH 7.5 containing 3 mM EDTA) to-

100

l were used for each time point. For determination of the thiol
l of phosphate-

Plasmid Description (reference)

pTL110 YEp352 containing the ADH promoter as a 260-bp Sall-SphI fragment (3, 17)
pTL111 pTL110 containing the complete gene for plant AtErv1 from A. thaliana cloned into the SmaI site behind the
ADH promoter (this study)
pET24a(+) Expression vector for His₆-tagged proteins (Novagen)
pUC19 Vector for cloning and sequencing of DNA fragments (18)
pAVA321 Vector for fusion of GFP carboxyl terminus with amino terminus of AtErv1 (19)
pUC19 Vector for fusion of the fusion of AtErv1 carboxyl terminus with amino terminus of GFP (20)
pBSC-preCytb₂ Vector for fusion of the import sequence of yeast Cytb₂ with AtErv1 (3)

Plasmids used in this study

Primer Sequence Description

Primer Description (reference)

AtErv1 (NdeI) 5'-CCCCAAAGATATGCGTGAGAAGCCATGCGGAC3' First possible ATG for AtErv1
rAtErv1 (XhoI) 5'-ACATCTGGAGAAGTCTAGAAGTTTGCAG3' Reverse primer for 3' end (stop codon) of AtErv1
rCytb₂-6H (KpnI) 5'-CCCGGTACCCTACGTTGGGTTGTTG3' Reverse primer for 3' end with His₆ tag and stop codon for amplification of AtErv1 from pET24a(+) 
rCytb₂ (KpnI) 5'-CCCGGGTACCCTAAAAGTCATGAAAGTTTGCAG3' Reverse primer for 3' end with natural stop codon of AtErv1
AtErv1-GFP (EcoRV) 5'-CCCCAAAGATATGCGTGAGAAGCCATGCGGAC3' First possible ATG for AtErv1
rAtErv1-GFP (SmaI) 5'-AACACCTATCCGCTGCGAACAAATCCTCCAAGGAC3' Reverse primer for 3' end (stop codon) of AtErv1
GFP-1AtErv1 (BamHI) 5'-CCCGGGTACCCTACGTTGGGTTGTTG3' Subcloning of AtErv1 BamHI-KpnI into YEp352-ADH and excision of AtErv1 as a BamHI-SacI fragment \( \rightarrow \) fusion with pAVA321 (BglII/SacI)
rDelta-1C (XhoI) 5'-TGGAACCTCGAGACTTTCTGCTCAGACTCTTAC3' Reverse primer for deletion of the first 10 amino acids with Cys\(\text{102}\)
rDelta-2C (XhoI) 5'-TCTGACCTCGAGCTTCAATGCCCCATCTTGC-3' Reverse primer for deletion of the last 15 amino acids with Cys\(\text{112}\)

Spectroscopy of Plant Erv1p-His₆—The isolated 15-kDa protein fol-
was grown on solid 

was excised as a BamHI/KpnI fragment and inserted into the compat-

yeast Cytb₂ with AtErv1 (3)

E. coli 

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Plants Growth, Protoplast Transformation, and Fluorescence Micros-
scopy—Arabidopsis protoplasts were isolated and transfected as de-
scribed in Ref. 26. Typically, \(2 \times 10^4\) protoplasts were transfected with 

was grown on solid 

Western Analysis—Non reducing SDS-PAGE (gradient gels of 4–12%) (Novex/Invitrogen) was used to separate 200-ng aliquots of 15-

was excised as a BamHI/KpnI fragment and inserted into the compat-

yeast Cytb₂ with AtErv1 (3)
was added to 300 μl of a protoplast suspension. Following gentle mixing, 300 μl of solution containing 40% polyethylene glycol, 0.1 M CaNO₃, 0.38 M mannitol, and 10 mM Tris-HCl, (pH 8.0) was added, and the newly identified plant sulfhydryl oxidase from Arabidopsis thaliana (EMBL accession number for AtErv1, AJ551263) harbors a carboxyl-terminal cysteine pair that is separated by 4 amino acid residues (C-4-C).

FIG. 1. Conserved regions and unique cysteine motifs in sulfhydryl oxidases from yeast, human and plant. A highly conserved region (gray bar) with about 40% identical residues in a domain of 100 amino acids is connected by the FAD-binding site, the catalytic core, and a cysteine pair that forms a structural disulfide bridge (bracket). These two cysteines are always exactly 16 amino acids apart (C-16-C). The amino- and carboxyl-terminal domains are not conserved but contain one additional cysteine pair. Location and arrangement of these cysteine pairs is specific for each enzyme. Erv2p form the endoplasmic reticulum is characterized by a unique CXXC motif in the carboxyl-terminal domain, whereas mitochondrial yeast Erv1p and human Alr contain a CXXC motif in the amino-terminal part. In contrast, the newly identified plant sulfhydryl oxidase from A. thaliana (Fig. 1) contains the redox center, the FAD-binding site, and a conserved catalytic core is highly conserved, whereas the amino-terminal cysteine pair separated by exactly four amino acids is important of the new CXXXX motif in Erv1 from higher plants.

In contrast, the highly conserved central core exhibits all features typical to members of the Erv1/Alr protein family. The available structural data (12) from the conserved central domain of yeast Erv2p and Erv2p (30, 31), the first crystallized member of the Erv1/Alr protein family (Protein Data Bank accession number 1J28), allowed the comparative modeling of AtErv1. The modeled structure of the conserved core from amino acids 76 to 176 of plant AtErv1 is presented in Fig. 3. A four-helix bundle forms the putative FAD-binding site, and the conserved catalytic cysteine pair, YPCXXC, is in close proximity to the isoalloxazine ring. A structural disulfide bridge between a second cysteine pair that is separated by exactly 16 amino acids in Erv1p, Alr, and Erv2p is also predicted for AtErv1. This disulfide bridge fixes the short fifth α-helix against the four helix-bundle and is important for stabilizing the FAD-binding pocket (12, 14).

Expression and Characterization of Recombinant Plant AtErv1—To define the functional characteristics of plant AtErv1 two recombinant proteins with a His₈ tag were synthesized in E. coli. The putative full-length protein of 22 kDa and the carboxyl-terminal domain of 15 kDa were successfully expressed. Analysis of the proteins on nonreducing SDS-PAGE demonstrated that both fragments form dimers and multimers (Fig. 4A), indicating that in contrast to the yeast protein, the amino terminus of AtErv1 does not participate in dimerization. Although the cause of multimerization of AtErv1 is yet unknown, it is not unique, because it is typically observed for all Erv1/Alr proteins. In vitro experiments show that multimerization of all these proteins is dependent on the concentration and the number of cysteine residues in mutant proteins (14). For the 22-kDa protein, degradation products were routinely found, indicating increased instability of this gene product. The 15-kDa fragment did not show any prominent degradation products. Therefore further characterization of AtErv1 was performed with the 15-kDa fragment that contained both the redox-active YPCXXC motif and the FAD-binding domain.

The disappearance of AtErv1 dimers upon reduction with diithiothreitol (Fig. 4A) indicated that as in Erv1p and Erv2p dimerization of AtErv1 is mediated by an intermolecular disulfide bridge. The conservation of the carboxyl-terminal CXXXX motif in all plant homologues of AtErv1 suggested that it functionally replaced the amino-terminal cysteine pair of Erv1p.

The fluorescence images were taken 24 h after transformation using a laser confocal microscope (Olympus Fluoview FV500). The images were obtained using an excitation wavelength of 488 nm, with GFP and chlorophyll images collected through 505–525- and 630-nm filters, respectively. Mitochondria were visualized using the mitochondrial-specific dye MitoTracker (Molecular Probes), with excitation of 543, dichroism of 543, and emission long pass of 560 nm.

Miscellaneous Methods—E. coli strains DH5-α (15) and BL21 (Clontech) were used for cloning experiments and amplification of plasmid DNA. Plasmid DNA was isolated from E. coli by alkaline lysis (Qiagen kit). Transformation of yeast cells was done with lithium acetate according to a standard protocol (28). Purification, restriction enzyme digestion, ligation, and analysis of yeast DNA or PCR products on agarose gels were performed using standard techniques (29). Nucleotide sequences of the DNA constructs were determined by automatic DNA sequencing (SeqLab Company, Göttingen, Germany).

RESULTS

Cloning of the Plant cDNA for AtERV1—The first aim was to identify putative Erv1 homologues in higher plants. Screening of Arabidopsis sequence data bases with the yeast Erv1 protein sequence identified only one nuclear gene as a possible candidate. The most reliable sequence data were used to synthesize primers for the putative full-length cDNA. These primers (Table III) were included in PCR experiments with an Arabidopsis cDNA library (Clontech). DNA fragments of about 600 bp were amplified. Three independent clones were isolated and subjected to DNA sequencing. All three DNA inserts exhibited identical DNA sequences. The cDNA (AtErv1) contained an open reading frame with the capacity to encode a protein of 191 amino acids (EMBL accession number AJ551263). The comparison of the putative plant protein with yeast Erv2p and yeast and human Erv1p/Alr is shown in Fig. 1. The central domain with the catalytic core is highly conserved, whereas the amino- and carboxyl-terminal regions reveal unique features.

Unique CXXXX Motif and Highly Conserved Central Core—Comparing the sequences of yeast, human, and plant sulfhydryl oxidases identifies a carboxyl-terminal CXXXX motif as a unique feature of AtErv1 (Fig. 1). Close inspection of the available data bases from the genome sequencing projects of higher plants identified several Erv1 homologues. Notably, a carboxyl-terminal cysteine pair separated by exactly four amino acids is present in all putative plant Erv1 homologues from dicots and monocots identified thus far (Fig. 2), arguing for the functional importance of the new CXXXX motif in Erv1 from higher plants.

FIG. 2. Alignment of the last carboxyl-terminal residues from AtErv1 with other plant Erv1 homologues. The conserved cysteines of the CXXXX motif are marked by arrows and underlined. The first five sequences are from dicots, and the last three are from monocots. Most of the sequences are preliminary data from genome sequencing projects, and therefore the carboxyl-terminal ends may not be complete. (The GenBank™ accession numbers are: Solanum tuberosum, 21917679; Vitis vinifera, 33409483; Medicago truncatula, 35105603; Glycine max, 7478576; Hordeum vulgare, 24227870; Sorghum bicolor, 15545857; and Zea mays, 37394272.)
determine whether the CXXXXC motif participates in an interdisulfide bridge, two mutant forms of AtErv1, one lacking the carboxyl-terminal member of the cysteine pair and a second missing the entire CXXXXC motif, were produced and purified from *E. coli*. The disappearance of dimer formation in the mutant form lacking the plant conserved carboxyl-terminal cysteine pair and the increased dimer and multimers formation in the single cysteine mutant protein (Fig. 4B) strongly argue for the functional replacement of the amino-terminal cysteine pair of Erv1p by the carboxyl-terminal CXXXXC motif in AtErv1.

The presence of FAD in the purified protein was verified by spectroscopy (Fig. 5). The spectrum of the 15-kDa fragment exhibited the characteristic features of a protein-bound FAD moiety. Assays for enzymatic activity using artificial in vitro substrates demonstrated sulfhydryl activities (Fig. 6) with turnover numbers comparable with those reported for the yeast mitochondrial enzyme (1, 2). Like the yeast protein, the plant enzyme did not use mercaptoethanol or glutathione as a substrate. Notably, AtErv1 exhibited a high preference for dithiol reductants and particularly for thioredoxin.

**Complementation Studies between Yeast erv-ts, Δerv1, and the Plant Enzyme**—The available mutants of yeast Erv1p (16) allowed in vivo complementation studies with plant Erv1p. For this purpose, the full-length cDNA for *AtERV1* was cloned into a yeast expression vector. Neither the erv1-ts mutant nor the deletion mutant could be rescued by the plant enzyme (Fig. 7). To exclude any defects in the import of the plant enzyme into
yeast mitochondria, a new set of experiments was initiated where the yeast import sequence from Cytb2 was fused to the amino terminus of AtErv1. This fusion protein also gave no detectable complementation activity in vivo. The presence of the His6 tag, which had been shown previously not to obstruct complementation with the yeast Erv1p or human Alrp proteins, allowed us to readily confirm that the fusion protein is correctly expressed in yeast and imported into mitochondria (data not shown). Thus, despite their sequence similarities, the yeast and plant homologues are also functionally distinct.

Localization Experiments in Plant Cells with Fusion Proteins of GFP and AtErv1—Analysis of the AtErv1 sequence with the current computer programs for predicting subcellular localization (MitoProt II, Predotar, PSORT II, SignalP) identified no typical import sequence for mitochondria and even predicted a possible plastid localization (data not shown). Therefore, it was essential to confirm the subcellular localization of this protein in vivo. Two constructs of GFP fused either to the carboxyl terminus (AtErv1-GFP) or the amino terminus of AtErv1 (GFP-AtErv1) were transiently expressed in plant protoplasts from Arabidopsis or Physcomitrella. Confocal fluorescence microscopy of protoplasts expressing the AtErv1-GFP construct revealed the accumulation of GFP in distinct, somewhat spherical, bodies localized outside the chloroplasts in both plants (Fig. 8). Staining the protoplasts expressing the AtErv1-GFP construct with the mitochondria-specific dye, MitoTracker, showed that the GFP fluorescence colocalized with the mitochondria. In contrast, the fluorescence of the reverse orientation GFP-AtErv1 fusion proteins appeared diffuse and outside the mitochondria and chloroplasts (Fig. 8), suggesting that the amino-terminal leader of AtErv1 is required for efficient targeting to the mitochondria.

DISCUSSION

In this communication we describe the cloning and characterization of AtErv1, the first mitochondrial FAD-linked sulfhydryl oxidase identified from a photosynthetic organism. The central core of the plant enzyme exhibits the characteristic structural and functional features of mitochondrial Erv1p/Alrp sulfhydryl oxidases, whereas the amino- and carboxyl-terminal regions show distinct differences.

Screening of genomic, cDNA, and protein data bases for A. thaliana identified only one possible candidate for a sulfhydryl oxidase homologous to yeast Erv1p (1). Cloning of the cDNA and sequence analysis demonstrated the presence of an open reading frame that could encode a 191-amino acid protein. The central domain showed perfect alignment with Erv1p/Alrp from yeast and mammals. Structural modeling revealed the highly conserved four-helix bundle for FAD binding, the close proximity of the conserved YPCXXC motif with the isoalloxazine ring of FAD, and a cysteine pair that participates in the structural alignment of the fifth short helix in a defined position (12, 13).

In contrast, the amino-terminal and the carboxyl-terminal domains of AtErv1 display no significant amino acid homologies to other family members. This is a common feature of all Erv1/Alrp proteins (8, 17). The most significant differences from other Erv1/Alrp proteins are the absence of a cysteine pair in the amino-terminal domain of the plant enzyme and the presence of a unique carboxyl-terminal cysteine pair. For the yeast enzyme, it was shown that the amino-terminal cysteine pair is essential for dimerization and interdomain redox communication, and thus this cysteine pair is indispensable for its in vivo function (14). The functional replacement of such an amino-terminal cysteine pair by a carboxyl-terminal GCC motif has recently been described for Erv2p, a second yeast sulfhydryl oxidase that is localized in the endoplasmic reticulum (12).

Moreover, the additional amino- or carboxyl-terminal cysteine pairs that transfer disulfides, initially generated at the primary YPCXXC motif, appear to be a unique hallmark of the enzymatic mechanism of sulfhydryl oxidases bearing the Erv1p/Alrp domain (11). Finally, an additional indication for the functional conservation of an additional cysteine pair in all mitochondrial Erv1/Alrp proteins is illustrated by recent studies of avian QSOX, demonstrating that the formation of a divalent metal ion-binding site by the Erv1/Alrp domain and a second CXXC motif requires the two cysteine pairs (32).

It should be noted, however, that so far the physiological role of metal binding by QSOX or Erv1p/Alrp has yet to be addressed experimentally. The disulfide transfer reaction by the amino- or carboxyl-terminal cysteine pair is important for the interaction with substrate molecules or peptides in vivo (14). In this respect it is highly significant that the plant enzyme contains a new cysteine pair near its carboxyl terminus that functionally replaces the intermolecular disulfide-mediated dimerization of the protein by the amino-terminal cysteine pair in Erv1p and Alrp, because this might indicate that the plant enzyme developed an alternative mechanism for the transfer of the disulfide bond from the primary redox center to possible mitochondrial substrates. The strict conservation of the carboxyl-terminal cysteine pair with always exactly four amino acids between these two residues in the available Erv1 sequences of all spermatophyta is a further indication for a unique specificity of the plant protein.

The specific structural feature of the CXXXXC motif at the carboxyl terminus of the plant enzyme is a plausible explanation why the plant enzyme cannot substitute for the yeast enzyme in vivo despite its highly conserved catalytic core. Even enforced mitochondrial import of the plant enzyme into the intermembrane space of yeast mitochondria via the highly efficient pre-Cytb2 import sequence did not result in functional complementation. Nevertheless, it is highly likely that plant AtErv1 and yeast Erv1p, which clearly evolved from the same ancestral enzyme and are both localized to the mitochondria, share similar organelar functions. Yet the more complex requirements in a photosynthetic multicellular organism may have resulted in adaptation of AtErv1 to new substrates and functions that are not present in the unicellular yeast.

Our experiments demonstrate that the targeting of full-length Arabidopsis plant enzyme to mitochondria is conserved because it also efficiently localized in vivo to mitochondria of a
lower plant, *Physcomitrella*. The failure of the current computer programs to identify a mitochondrial import sequence for AtErv1 is not surprising in light of the fact that proteins of the mitochondrial intermembrane space have individual and peculiar import pathways and therefore do not exhibit a conserved consensus targeting sequence (33). Furthermore, none of the known mitochondrial Erv1/Alr proteins show any detectable processing of the amino terminus after import into mitochondria (2, 3, 34). Nevertheless, although we found no experimental evidence that AtErv1 is localized to plastids, it cannot be ruled out that a small fraction of AtErv1 is also directed toward this compartment.

Our data show that the central core of AtErv1 has all the characteristic structural and functional properties of mitochondrial Erv1p/Alrp sulfhydryl oxidases. AtErv1 forms homodimers, and similarly to yeast Erv1p, its *in vitro* activity shows a high preference for dithiol reductants, in particular for reduced thioredoxin, over monothiol reductants, such as cysteine, glutathione, and mercaptoethanol. Hence, it is plausible that a dithiol oxidoreductase, such as thioredoxin or protein disulfide isomerase, is an essential redox partner for the mitochondrial Erv1p. An additional argument for functional interactions between Erv1p/Alrp enzymes and thioredoxin-like proteins is suggested by the structure of the QSOX enzymes, in which the ERV1 domain was physically linked with a thioredoxin domain during evolution. Notably, the QSOX enzymes

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**Fig. 7.** Complementation studies with yeast erv-ts, Δerv1, and plant AtErv1. The proteins shown were expressed in mutant yeast cells with a temperature-sensitive Erv1p (erv1-ts) or a deleted copy of the gene (Δerv1). Controls transfected with the wild-type yeast protein resulted in complementation, whereas plant Erv1p either containing or lacking a His6 affinity tag at its carboxyl end was not able to substitute for the yeast protein. To exclude any mislocalization of the plant protein, a fusion protein with the yeast-specific intermembrane space targeting sequence of Cytb2 (pCytb2) was included in these experiments.

**Fig. 8.** AtErv1-GFP fusion protein is targeted to mitochondria *in vivo*. Constructs of AtErv1 fused either at the carboxyl termini (AtErv1-GFP) or at the amino termini (GFP-AtErv1) to GFP were introduced into protoplasts from both *A. thaliana* (A) and *P. patens* (B). Fluorescence images of protoplasts show the green fluorescence of GFP fusion proteins (GFP), the red fluorescence of mitochondria stained with MitoTracker (mito), or the red auto-fluorescence of chloroplasts (chpst) and below the combined image (merge).
exhibit dramatically enhanced enzymatic activity (9), and its truncated form lacking the thioredoxin-like domain displayed 1000-fold slower activity (11). Thus, it is interesting to notice that a recent study identified a mitochondrial thioredoxin system in plants (35).

One specific essential function of Erv1p in yeast concerns the presence of an iron/sulfur cluster assembly mechanism inside mitochondria, which is also required for cytosolic iron/sulfur protein assembly (36). Genetic and biochemical experiments suggested that yeast Erv1p interacts with the ABC transporter Atm1p of the inner mitochondrial membrane for the export of iron/sulfur clusters from mitochondria (3). Recent studies identified the first components of an iron/sulfur assembly machinery in plant mitochondria and demonstrated that the plant mitochondrial ABC transporter, Sta1, is a functional homologue in plant mitochondria and demonstrated that the plant iron/sulfur clusters from mitochondria (35). It will be interesting to identify the specific adaptations of the AtErv1 enzyme to the requirements of plant mitochondria.

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