An Arabidopsis indole-3-butyric acid-response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function

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Edited by Joanne Chory, The Salk Institute for Biological Studies, La Jolla, CA, and approved December 9, 2003 (received for review July 11, 2003)

Genetic evidence suggests that plant peroxisomes are the site of fatty acid β-oxidation and conversion of the endogenous auxin indole-3-butyric acid (IBA) to the active hormone indole-3-acetic acid. Arabidopsis mutants that are IBA resistant and sucrose dependent during early development are likely to have defects in β-oxidation of both IBA and fatty acids. Several of these mutants have lesions in peroxisomal protein genes. Here, we describe the Arabidopsis pex6 mutant, which is resistant to the inhibitory effects of IBA on root elongation and the stimulatory effects of IBA on lateral root formation. pex6 also is sucrose dependent during early seedling development and smaller and more pale green than WT throughout development. PEX6 encodes an apparent ATPase similar to yeast and human proteins required for peroxisomal biogenesis, and a human PEX6 cDNA can rescue the Arabidopsis pex6 mutant. The pex6 mutant has reduced levels of the peroxisomal matrix protein receptor PEX5, and pex6 defects can be partially rescued by PEX5 overexpression. These results suggest that PEX6 may facilitate PEX5 recycling and thereby promote peroxisomal matrix protein import.

In eukaryotes, peroxisomes serve to compartmentalize several metabolic processes, including fatty acid β-oxidation and H2O2 inactivation by catalase (1). In plants, leaf peroxisomes act with chloroplasts and mitochondria in photosynthesis (1) and catalysis of branched-chain amino acids (2), whereas specialized peroxisomes called glyoxysomes contain glyoxylate cycle enzymes (1, 3, 4). Plant peroxisomes also are implicated in developmental events, including photomorphogenesis (5) and lateral root formation (6), and jasmonic acid synthesis (7) required for wounding responses (7).

Identification of genes altered in peroxisome-defective yeast mutants and humans with peroxisomal biogenesis disorders has implicated >20 peroxin (PEX) proteins in peroxisomal biogenesis and function (8–10). Phenotypic characterization of the mutants and biochemical studies of the proteins have linked many PEXs to particular aspects of peroxisome biogenesis, maintenance, and matrix protein import.

Two peroxisomal targeting signals (PTSs) direct matrix-bound proteins from the cytoplasm into peroxisomes: PTS1 consists of Ser-Lys-Leu (SKL), or related variants, at the extreme C terminus of the protein, whereas PTS2 is a nonapeptide found near the N terminus (1, 9, 11). Proteins destined for the peroxisomal matrix are bound by the PEX5 or PEX7 receptors in the cytoplasm and escorted into peroxisomes (1, 9, 11). Recent evidence suggests that the receptor–matrix protein complex dissociates in the matrix after import, releasing the matrix proteins, and the receptors are recycled to the cytoplasm for another round of import (11, 12). Certain PEXs are clearly implicated in docking of the receptor–matrix protein complex at the membrane and translocation into the matrix (1, 8, 9), whereas the roles of others are less well defined. For instance, PEX1 and PEX6 are interacting ATPases required for matrix protein import (13–15); the exact role of these proteins in peroxisomal function, however, remains unclear.

Much of what is known about plant peroxisome biogenesis is based on similarities to yeast or human systems. Sequence comparisons indicate that many, but not all, PEX proteins have plausible homologs in Arabidopsis (8, 16), and several Arabidopsis PEX proteins have been described (5, 17–24). The identification of Arabidopsis peroxisome-defective mutants confirms the importance of plant peroxisomal functions and provides an unbiased method to identify important peroxisomal proteins (25, 26).

In this work, we demonstrate that a defect in the Arabidopsis PEX6 gene disrupts IBA responses and has a strong peroxisome-defective phenotype. PEX6 encodes an apparent ATPase similar to yeast and human proteins necessary for peroxisomal function. Our analysis of the pex6 mutant indicates that PEX6 may facilitate PEX5 recycling.

Materials and Methods

Phenotypic Analysis. pex6 was identified in an IBA-response screen of ethyl methanesulfonate-mutagenized Columbia (Col-0) seeds; the mutant was originally designated B11 (24). Seeds were surface-sterilized (28) and plated on plant nutrient medium (PN) (29) solidified with 0.6% agar and containing sucrose and hormones as indicated. All phenotypic assays were conducted at least twice with similar results on backcrossed mutant lines.

Genetic Analysis and Mutant Complementation. pex6 was outcrossed to Wassilewskija for mapping. F2 seedlings were grown on 15 μM IBA, DNA was isolated from resistant individuals (30), and the defect was mapped by using published markers (31). A candidate gene (PEX6, At1g03000) was PCR-amplified from mutant DNA, and overlapping fragments covering the gene from 145 bp upstream of the putative translation start site to 193 bp downstream of the stop codon were sequenced.

A PEX6 genomic clone was constructed by subcloning a 6.6-kb XmaI fragment (1.8-kb 5′ UTR and 0.4-kb 3′ UTR) from bacterial artificial chromosome F10O3 into Smal-cut pBluescript II KS+. A BamHI/SalI fragment containing PEX6 was subcloned into the plant transformation vector pBIN19 (32) cut with the same enzymes to give pBIN-PEX6. The 35S-HsPEX6 clone was constructed by subcloning a SalI/XhoI fragment containing a full-length human...
**PEX6 cDNA (L.M.A.G.E. Consortium, Lawrence Livermore National Laboratory, Berkeley, CA, clone ID 5140908) into the plant transformation vector 35S:BGAR (33) cut with Xhol and NotI.** pBIN-PEX6, 35S-HsPEX6, and 35S-PEX5 (24) were electroporated into *Agrobacterium tumefaciens* strain GV3101 (34) and transformed into *pex6* plants (35). Transformants were selected for the ability to develop on PN in the dark or PN containing 15 mM sucrose plus 12 μg/ml kanamycin or 7.5 μg/ml glufosinate ammonium (Basta), and homozygous progeny were used for phenotypic assays.

**GFP Analysis.** We obtained a cytoplasmically targeted GFP construct optimized for plant use (36, 37). To make a peroxisome-targeted line, we performed oligonucleotide-directed mutagenesis (38) with the oligonucleotide 5′-GGATGAATATACA-AAGCAAGCTTTAAGACGTACATTCCTCC-3′, inserting a C-terminal peroxisomal signal “SKL” (underlined) immediately preceding the termination codon (bold). The GFP-SKL gene was subcloned into the pBICaMV vector (39) behind the constitutive 35S promoter to give 35S-GFP-SKL.

The GFP construct was transformed into WT Col-0 plants. Homozygous transformants were crossed to the *pex6* and *pxa1* (6) mutants and homozygous *pex6* (pBIN-PEX6) rescue plants. To examine GFP expression, seeds were plated on PN with 15 mM sucrose and incubated under white light overnight, then for 4 days in the dark. Whole plants were mounted on slides, and GFP localization in seedling root hairs was examined by using a Zeiss Axioscan 2 fluorescence microscope equipped with a narrow-band GFP filter set (41020, Chroma Technology, Rockingham, VT).

**PEX5 Protein Analysis.** Seeds were surface-sterilized and placed under white light for 2 days in water. Protein samples were prepared by grinding seedlings on ice with a motorized pestle, adding an equal volume of 2× NuPAGE LDS-sample buffer with reducing agent (Invitrogen), and heating to 80°C for 10 min. Proteins were separated by gel electrophoresis using NuPAGE 10% Bis-Tris gels and transferred at 24 V for 1 h to Hybond ECL nitrocellulose membrane (Amersham Pharmacia) by using NuPAGE transfer buffer according to the manufacturer’s instructions.

A rabbit antibody (α-PEX5) was generated against the C-terminal region of PEX5 (A713-L728, ACESRNLDLLOKEFPL) and affinity-purified by Bethyl Laboratories (Montgomery, TX). For immunoblotting, membranes were incubated in blocking buffer (8% milk in TBS-T) (38) for 2 h, then with α-PEX5 (0.05 mg/ml in blocking buffer) overnight at 4°C, followed by horseradish peroxidase (HRP)-linked goat α-rabbit IgG (Santa Cruz Biotechnology) diluted 1:500 in blocking buffer for 1 h. Control blots used α-HSC70 (SPA-795; StressGen Biotechnologies, Victoria, Canada) at a 1:1,500 dilution. HRP was visualized by using LumiGLO reagent (Cell Signaling Technology, Beverly, MA).

**Results**

The *pex6* Mutant Displays Peroxisome-Defective Phenotypes. *pex6* was identified in a screen for IBA-response mutants (24). The mutant is resistant to root elongation inhibition by IBA (Fig. L4), but responds normally to indole-3-acetic acid (IAA) (24). Moreover, *pex6* fails to respond to the stimulatory effects of IBA on lateral root initiation (Fig. 1B), but makes lateral roots in response to IAA (24). These phenotypes indicate that *pex6* is not generally defective in lateral root initiation or auxin responsiveness, but is specifically defective in IBA responses.

Like a subset of IBA-response mutants (6, 24, 27), *pex6* displays peroxisome-defective phenotypes. In oilseed plants like *Arabidopsis*, long-chain fatty acids stored in seeds are β-oxidized during germination to provide energy; mutants defective in peroxisomal β-oxidation do not develop normally without exogenous sucrose (40). Whereas WT plants develop similarly with and without sucrose, *pex6* has dramatic defects in hypocotyl and root elongation in the absence of sucrose, but partially recovers when sucrose is supplied (Fig. 1 C and D). A more severe β-oxidation mutant, *pxa1* (6), is shown for comparison. Both the IBA resistance and sucrose dependence of *pex6* are recessive (data not shown).

*pex6* also has obvious seedling and adult phenotypes. Even with...
supplemental sucrose, the mutant seedling has a shorter root (Fig. 1D) and hypocotyl (data not shown) than WT. *pex6* plants grown in soil have smaller rosettes, fewer rosette leaves, and shorter primary inflorescence stems than WT (Fig. 2 and Table 1). In addition, the mutant has shorter siliques (Fig. 2F) containing fewer seeds than WT, resulting in decreased fecundity. The mutant is pale green in color (Fig. 2), similar to mutants defective in photosynthesis, a peroxisomal process requiring PTS1 import (41). Similarly reduced pigmentation is seen in the *ped2*/*ped4* mutant (18) but not in *pxa1*, which is defective in a peroxisomal transporter but not matrix protein import (6).

**Positional Cloning of the Gene Defective in *pex6***. Using recombination mapping, we localized the gene defective in *pex6* to the top of chromosome 1 (Fig. 3A). Within this region, we identified a candidate gene (At1g03000) that encodes a protein resembling human (42) and sunflower (19) PEX6 proteins, which are ATPases previously implicated in peroxisomal function. Sequencing this gene from mutant genomic DNA revealed a G-to-A mutation that replaces a conserved Arg with a Gln residue (Fig. 3C; Table 1). To verify that the nucleotide change in the *pex6* mutant causes the IBA-response mutant phenotype, we transformed a genomic fragment containing the WT *PEX6* gene including its promoter into mutant plants and assessed complementation. This construct (pBIN-*PEX6*) alleviates the *pex6* growth defects (Figs. 1C and D) and restores IBA sensitivity to root elongation (Fig. 1A) and lateral root initiation (Fig. 1B), confirming that we have identified the gene responsible for the *pex6* phenotypes.

**Human PEX6 Functionally Complements the Arabidopsis *pex6* Mutant.** Fig. 3C shows a partial alignment of the *Arabidopsis* PEX6 with homologs from other organisms. To determine whether the human and plant proteins function similarly, we transformed *pex6* mutant plants with a human PEX6 cDNA driven by the cauliflower mosaic virus 35S promoter. This construct rescued the *pex6* phenotypes similarly to the genomic *Arabidopsis PEX6* construct, restoring WT sensitivity to IBA in root elongation (Fig. 1A) and lateral root initiation (Fig. 1B), imparting sucrose independence during seedling development (Fig. 1C and D), and rescuing the size and pigmentation defects of mature *pex6* plants (Fig. 2B and D).

**Visualizing *pex6* Peroxisomes.** To visualize peroxisomes in the *pex6* mutant, we examined expression of a GFP containing a PTS

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**Table 1. *pex6* growth defects**

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th>Rosette leaves, n</th>
<th>Rosette diameter, cm</th>
<th>Inflorescence height, cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col-0</td>
<td><em>pex6</em></td>
<td>Col-0</td>
</tr>
<tr>
<td>17</td>
<td>9.2 ± 0.2</td>
<td>8.3 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>25</td>
<td>16.3 ± 0.9</td>
<td>11.2 ± 0.4</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>32</td>
<td>34.3 ± 1.6</td>
<td>17.5 ± 1.3</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>41</td>
<td>41.8 ± 1.6</td>
<td>21.7 ± 1.6</td>
<td>6.4 ± 0.2</td>
</tr>
</tbody>
</table>

WT Col-0 and *pex6* were grown on hormone-free medium with 15 mM sucrose for 10 days, then transferred to soil. Numbers represent the means ± standard errors (n = 9).
Reduced Levels of the Matrix Protein Receptor PEX5 in the pex6 Mutant. The precise role of PEX6 in peroxisomal processes has not been determined in any system. Immunofluorescence studies indicate that human fibroblast and Pichia pastoris pex6 mutants have reduced PEX5 levels (42, 45, 46). One hypothesis is that PEX6 functions in recycling PEX5 to the cytoplasm after peroxisomal matrix protein import. This hypothesis predicts that in a pex6 mutant PEX5 might perform one round of import and then be trapped in the peroxisome, where it is sequestered or degraded. Therefore, in the pex6 mutant, PEX5 would have limited function, resulting in slowed matrix protein import and peroxisome-defective phenotypes. To determine whether the Arabidopsis pex6 mutant has reduced PEX5 levels, we generated a peptide antibody that recognizes PEX5 and compared PEX5 levels in 2-day-old WT, pex6, and pex6 lines transformed with either the pBIN-PEX5 genomic construct or a 3SS-PEX5 overexpression construct. We found that the Arabidopsis pex6 mutant has reduced PEX5 protein levels (~10% of WT; Fig. 5A). Transforming the mutant with either the genomic PEX6 construct or the PEX5 overexpression construct increased PEX5 levels (Fig. 5A).

To determine whether restoring PEX5 levels in pex6 could restore peroxisomal functions, we examined the phenotypes of pex6 plants expressing 35S-PEX5. This construct partially rescued pex6 growth defects, including hypocotyl and root length (Fig. 5B) and the adult fertility, size, and pigmentation phenotypes (Fig. 2A and E), suggesting that PEX5 becomes limiting in the pex6 mutant. Interestingly, PEX5 overexpression did not restore normal sensitivity to IBA (data not shown), consistent with previous observa-
tions that IBA responses are more stringent measures of peroxi-
somal function than sucrose dependence (6).

Discussion

We have identified an Arabidopsis pex6 mutant in a screen for
IBA-response mutants. pex6 requires sucrose during early de-
velopment (Fig. 1 C and D), reflecting slowed rates of peroxi-
somal fatty acid β-oxidation (24). In addition, pex6 is resistant to
the inhibitory effects of IBA on root elongation and the stim-
ulatory effects of IBA on lateral root initiation (Fig. 1 A and B),
presumably reflecting defects in peroxisomal IBA β-oxidation.
Interestingly, whereas the sucrose dependence of pex6 may be
weaker than the peroxisome-defective mutant pxa1 (Fig. 1C),
pex6 has more obvious developmental delays and pigmentation
and fertility defects (Fig. 2 and Table 1) than pxa1 (6). These
phenotypes suggest globally defective peroxisomal function
in the mutant and reveal roles for PEX6 throughout plant
development.

Positional cloning of the gene defective in the mutant revealed a
missense mutation in PEX6 (Fig. 3). Arabidopsis PEX6 is 30%
identical to the previously characterized human PEX6, which can
functionally complement the Arabidopsis pex6 mutant defects (Fig.
1). PEX6 contains an AAA module, found in ATPases associated
with various cellular activities. AAA proteins comprise a distinct
subset of ATPases that may act in protein folding or as protein-
linked membrane protein clamps and have widely diversified roles
including protein sorting, vesicular secretion, and cell division
(47, 48).

Yeast have ~200 apparent ATPases defined by the AAA
module, a 200- to 250-aa motif containing an ATP-binding domain
and an adjacent consensus region (Fig. 3C and refs. 47-49).
AAA-domain proteins fall into 17 subfamilies; subfamily 2 proteins
have been implicated in peroxisomal processes (49). Arabidopsis
PEX6 and other subfamily 2 members contain two AAA modules;
similar to other PEX6 proteins (49), only the second module has
canonical Walker A and B ATP-binding site motifs. Fig. 3C shows
an alignment of this second conserved region with Arabidopsis,
rice, human, and yeasts proteins. Within this interval, Arabidopsis PEX6
is 74% identical to human PEX6, compared to 30% over the whole
protein. The pex6 mutation alters an Arg residue immediately after
the Walker B box (Fig. 3C) that is present in all characterized PEX6
proteins and numerous other AAA proteins (49).

The PEX6-interacting protein PEX1 is a second AAA protein
required for peroxisome biogenesis in yeast and mammals (13-15).
A cDNA encoding a PEX1-like protein (At5g08470) has been
cloned from Arabidopsis (21). At3g01610 encodes a second Arabi-
dopsis protein similar to PEX1 in the AAA module but more similar
to CDC48 overall. Like PEX6, these proteins have two
AAA domains, but the sequences are more diverged outside of this
region. Phylogenetic analysis reveals that Arabidopsis PEX6 is more
closely related to PEX6 proteins from other organisms than to other
Arabidopsis AAA proteins or to PEX1 proteins from other
organisms (Fig. 3D).

In humans, PEX6 disruption is lethal. Human fibroblasts defec-
tive in PEX6 and yeast pex6 mutants synthesize peroxisomal mem-
branes and import peroxisomal membrane proteins, but are defec-
tive in matrix protein import of both PTS1 and PTS2 proteins (42,
50). In addition, human fibroblasts and P. pastoris pex6 mutants have
either ~5-15% of WT PEX5 levels because of an increased
PEX5 degradation rate (42, 45, 46), suggesting that PEX6 may act
with PEX5 in peroxisomal matrix protein import. However, human
PEX5 and PEX6 proteins do not physically associate in vitro (42).

Based on the defects in matrix protein import and the decreased
PEX5 levels in pex6 mutants, PEX6 has been hypothesized to act
in the final steps of peroxisomal matrix protein import (45). PEX6
is proposed to act in recycling PTS receptors from the peroxisome
to the cytoplasm after each round of import (12, 42, 45, 46). This
proposed role is similar to that of the CDC48 AAA protein, which

Fig. 4. GFP-SKL localization in the pex6 mutant. Root hairs of 5-day-old
dark-grown seedlings were examined by using fluorescence microscopy for ex-
pression of a peroxisomally targeted GFP (GFP-SKL) in WT (A), pex6−/− (B), pex6−/− (C),
and pex6 (pBIN-PEX6) rescue lines (D). All images are shown at the same magni-
fication. (Scale bar: 25 μm.)

Fig. 5. PEX5 expression in the pex6 mutant. (A) PEX5 protein levels are
decreased in pex6. Western blotting using anti-PEX5 (Upper) to examine PEX5 levels or
anti-HSC70 (Lower) as a loading control was performed on protein from 2-day-old
WT (Col), pex6, pex6 (pBIN-PEX6), three homozygous pex6 lines expressing 35S-
PEX5 (D4, D14, and Z31), and Col-0 (35S-PEX5) seedlings. The pex6-2X lane was
loaded with 2 vol of protein. Positions of molecular mass markers (kDa) are
indicated on the left. (B) Overexpression of PEX5 can partially rescue the sucrose
dependence and root elongation defects of pex6. Lines from A were examined for
hylocotyl elongation in the dark in the absence of sucrose (filled bars) or root
elongation in the light on 15 mM sucrose (open bars) as described in Fig. 1. Error
bars indicate SEM (n = 13).
is required for protein retrotranslocation out of the ER (51). CDC48, PEX1, and PEX6 are approximately equally diverged from each other (Fig. 3D) and are more closely related to one another than to other AAA proteins (49).

Because some AAA proteins act in membrane fusion, an alternative hypothesis is that PEX6 acts in membrane fusion events during peroxisomal formation and enlargement (52). Yarrowia lipolytica pex6 mutants have mislocalized peroxisomal membrane proteins and accumulate ER membranes and small peroxisomal vesicles (52). Although PEX6 antibodies can inhibit fusion events in vitro, analysis of yeast deletion mutants indicate that PEX6 is not essential for membrane fusion (53).

These results are consistent with previous analyses of peroxisomal size and abundance in human fibroblasts defective in PEX6, where fewer, larger peroxisomes were observed (43, 44). In addition, this phenotype was seen in several other mutants with matrix protein import defects (43). An alternate explanation for the pex6 peroxisome defects is that peroxisome biogenesis is defective because of membrane fusion defects. However, if peroxisomes were not made or were enlarged because of a pex6 defect in membrane fusion, it seems unlikely that PEX5 overexpression would rescue pex6.

The diverse phenotypes of the Arabidopsis pex6 mutant suggest PEX6 importance in a variety of peroxisomal functions, including fatty acid and IBA β-oxidation in glyoxysomes and photosynthesis in leaf peroxisomes. Interestingly, null mutations of Arabidopsis PEX2 or PEX10 genes confer embryonic lethality (5, 54, 55), suggesting that functioning peroxisomes are required for embryogenesis. Although a sequence-tagged insertion mutant collection (56) reports a putative PEX6 insertion (SALK.087302), we were unable to verify the presence of this insert (unpublished work); isolation of null pex6 alleles will require other approaches. Regardless of the phenotype of the pex6 null allele, the viable but peroxisome-defective pex6−/− allele described here will be a valuable tool to further elucidate the molecular mechanism of PEX6 function and peroxisomal processes in plants.

We thank Ilineela Silva for assistance characterizing pex6 adult defects, the Arabidopsis Biological Resource Center (Ohio State University, Columbus) for the GFP and F10O3 clones, and Raquel Adham, Melanie Monroe-Augustus, Rebekah Rampey, and Andrew Woodward for critical comments on the manuscript. This work was supported by National Science Foundation Grants IBN-9982611 and 0315596 and Robert A. Welch Foundation Grant C-1309.