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The *Arabidopsis* Peroxisomal Targeting Signal Type 2 Receptor PEX7 Is Necessary for Peroxisome Function and Dependent on PEX5

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Plant peroxisomal proteins catalyze key metabolic reactions. Several peroxisome biogenesis *PEROXIN* (*PEX*) genes encode proteins acting in the import of targeted proteins necessary for these processes into the peroxisomal matrix. Most peroxisomal matrix proteins bear characterized Peroxisomal Targeting Signals (PTS1 or PTS2), which are bound by the receptors PEX5 or PEX7, respectively, for import into peroxisomes. Here we describe the isolation and characterization of an *Arabidopsis* peroxin mutant, *pex7-1*, which displays peroxisome-defective phenotypes including reduced PTS2 protein import. We also demonstrate that the *pex5-1* PTS1 receptor mutant, which contains a lesion in a domain conserved among PEX7-binding proteins from various organisms, is defective not in PTS1 protein import, but rather in PTS2 protein import. Combining these mutations in a *pex7-1 pex5-1* double mutant abolishes detectable PTS2 protein import and yields seedlings that are entirely sucrose-dependent for establishment, suggesting a severe block in peroxisomal fatty acid β-oxidation. Adult *pex7-1 pex5-1* plants have reduced stature and bear abnormally shaped seeds, few of which are viable. The *pex7-1 pex5-1* seedlings that germinate have dramatically fewer lateral roots and often display fused cotyledons, phenotypes associated with reduced auxin response. Thus PTS2-directed peroxisomal import is necessary for normal embryonic development, seedling establishment, and vegetative growth.

INTRODUCTION

Peroxisomes are organelles housing diverse and vital processes. Plant peroxisomes contain enzymes for photorespiration (Liepman and Olsen, 2001; Reumann, 2002) and are the primary, if not exclusive, site of fatty acid β-oxidation (Gerhardt, 1992; Kindl, 1993; Graham and Eastmond, 2002). In addition, plant peroxisomes are necessary for jasmonic acid (JA) biosynthesis (Wasternack and Hause, 2002) and are implicated in the conversion of indole-3-butyric acid (IBA) into the active auxin indole-3-acetic acid (IAA; Zolman *et al.*, 2000; Bartel *et al.*, 2001).

Peroxisome matrix proteins must be imported from the cytosol because peroxisomal proteins are encoded by nuclear genes. Import of matrix proteins is accomplished by an array of PEROXIN (PEX) proteins that function in matrix protein import or general peroxisome assembly. Although >30 *pex* mutants have been identified in yeast and mammals, in plants, only *pex2* (Hu *et al.*, 2002), *pex5* (Zolman *et al.*, 2000), *pex6* (Zolman and Bartel, 2004), *pex10* (Schumann *et al.*, 2003; Sparkes *et al.*, 2003), *pex14* (Hayashi *et al.*, 2000), and *pex16* (Lin *et al.*, 1999) *Arabidopsis* mutants have been isolated. However, plausible *Arabidopsis* homologues for many of the *PEX* genes remaining to be characterized have been identified through sequence homology (Mullen *et al.*, 2001; Charlton and López-Huertas, 2002).

Two sequences sufficient to signal matrix protein import into peroxisomes have been identified. One is the extreme C-terminal amino acid sequence Ser-Lys-Leu (SKL), or a conserved variant, designated Peroxisomal Targeting Signal type 1 (PTS1; Gould *et al.*, 1989; Mullen, 2002; Neuberger *et al.*, 2003; Reumann, 2004). The PEX5 receptor recognizes and binds cytosolic PTS1-containing proteins. Mutations in human *PEX5* can cause the peroxisome biogenesis disorders Zellweger syndrome and neonatal adrenoleukodystrophy (Dodt *et al.*, 1995). The PEX5-PTS1 complex binds a PEX14-PEX13 receptor complex at the peroxisome membrane (Albertini *et al.*, 1997) and is translocated into the peroxisome matrix (Dammai and Subramani, 2001) in a process dependent on PEX2, PEX10, and PEX12 (Dodt and Gould, 1996; Chang *et al.*, 1999). In the peroxisome matrix, PEX5 releases its cargo and is recycled to the cytosol (Dammai and Subramani, 2001) in a process dependent on PEX1, PEX4, PEX6, and PEX22 (Collins *et al.*, 2000).

An alternate sequence is the N-terminal Peroxisomal Targeting Signal type 2 (PTS2) composed of Arg-Leu-$(X)_5$-His-Leu or a number of variants within the first ~30 amino acids (Osumi *et al.*, 1991; Flynn *et al.*, 1998; Reumann, 2004). PEX7 is the receptor for PTS2-containing proteins (Rehling *et al.*, 1996). Mutations in human *PEX7* can cause the peroxisome biogenesis disorders Refsum disease (van den Brink *et al.*, 2003) and rhizomelic chondrodysplasia punctata (Braverman *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997). In *Saccharomyces cerevisiae*, Pex7p interacts with Pex18p and Pex21p, two functionally redundant proteins necessary for PTS2 protein import (Purdue *et al.*, 1998). In *Neurospora crassa* and *Yarrowia lipolytica*, Pex18p and Pex21p are replaced with the single Pex7p docking protein Pex20p (Sichting *et al.*, 2003; Einwächter *et al.*, 2001).

Mammals and plants appear to lack *PEX18*, *PEX20*, and *PEX21* orthologues. Mammalian PEX7 function instead depends on interaction with an isoform of PEX5 (PEX5L;

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Braverman *et al.*, 1998) containing a PEX7-binding domain (Matsumura *et al.*, 2000; Dodt *et al.*, 2001). Whereas most PEX5 mutants are defective in PTS1 protein import, a PEX5 mutant Chinese hamster ovary (CHO) cell line is specifically deficient in PTS2 protein import because of inability to bind PEX7 (Matsumura *et al.*, 2000). Similarly, *Arabidopsis* PEX7 interacts with PEX5 in vitro (Nito *et al.*, 2002), and in vitro import of a PTS2 protein into *Cucurbita pepo* glyoxysomes is enhanced by PTS1 protein addition (Johnson and Olsen, 2003). Indeed, a role for PEX7-PEX5 interaction in *Arabidopsis* PTS2-protein import has been proposed (Sparkes and Baker, 2002). The dependence of PEX7 on PEX5 interaction in some organisms suggests a convergence of function carried to the extreme in *Caenorhabditits elegans*, which lacks PEX7 entirely and uses a PTS1 on proteins targeted by PTS2 in other organisms (Gurvitz *et al.*, 2000; Motley *et al.*, 2000).

Insertional null mutations in *Arabidopsis pex2* (Hu *et al.*, 2002) and *pex10* (Schumann *et al.*, 2003; Sparkes *et al.*, 2003) confer embryo lethality, revealing the necessity of peroxisome function for early plant development. Plants heterozygous for the *AtPex10* knockout allele bear ~20% abnormal seeds that fail to germinate (Sparkes *et al.*, 2003); aborted seeds remain white rather than proceeding to green and contain embryos arrested at the late globular or heart stages of development (Schumann *et al.*, 2003). As peroxisomes are necessary for JA biosynthesis (Wasternack and Hause, 2002) and are implicated in the conversion of IBA into IAA (Bartel *et al.*, 2001), deficiencies in JA and IAA have been suggested as possible causes for *pex10* mutant lethality (Sparkes *et al.*, 2003). In addition, a double mutant defective in two peroxisomal acyl-CoA oxidase genes is embryo lethal (Rylott *et al.*, 2003), suggesting that β-oxidation is an essential function for which peroxisomes are required during embryogenesis.

Here we describe *pex7-1*, an *Arabidopsis* mutant defective in IBA response and PTS2 protein import into peroxisomes. In addition, we show that the previously isolated PTS1-receptor mutant *pex5-1* (Zolman *et al.*, 2000) is deficient in PTS2-, but not PTS1-protein import. Further, a *pex7-1 pex5-1* double mutant is severely deficient in peroxisome functions, requiring exogenous sucrose for growth, and displaying frequent embryonic abnormalities suggestive of reduced auxin levels or response.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

pex7-1 was isolated from the Col-0 T-DNA insertion line SALK_005354 (Alonso *et al.*, 2003) obtained from the ABRC and isolated using a PCR-based method. Amplification with the oligonucleotide PEX7–2 (5′-CTTCTCGAAGATTCAATTCAACGAT-3′) and the modified LBb1 (Alonso *et al.*, 2003) T-DNA left border primer LB1-Salk (5′-CAAACCAGCGTGGACCGCTTGCTGCA-3′) yielded a ~200-base pair product from mutant DNA, whereas PEX7–1 (5′-CTCGAATTTAGATTTCTCTCTCACTTTTA-3′) combined with PEX7–2 yielded a 252-base pair product in the presence of wild-type DNA, enabling genotypic determination. Combining either PEX7–1 or PEX7–2 with LB1-Salk yields a PCR product; both products were sequenced to determine the T-DNA/genomic DNA junctions, which are present at 95/96 and 98/99 base pairs upstream of the *PEX7* start codon. This result indicates an insertional event that resulted in T-DNA left border sequences oriented in both directions, with the T-DNA replacing a 3-base pair deletion from 96 to 98 base pairs upstream of the *PEX7* start codon. The *pex5-1* mutant in the Col-0 background contains an ethyl methanesulfonate-induced missense mutation described previously (Zolman *et al.*, 2000).

Phenotypic analyses were performed on mutants backcrossed to Col-0 at least once. Seeds were surface-sterilized, stratified overnight at 4°C in 0.1% agar, and grown on plant nutrient medium (PN; Haughn and Somerville, 1986) with 0.5% sucrose (PNS) unless otherwise noted. For root elongation assays, 5 μM IBA was added to medium from a 100 mM ethanol stock, and plants were grown for 8 d in continuous light under yellow filters (25–45 μE m^{-2} sec^{-1}) to reduce the destruction of indolic compounds by short-wavelength light (Stasinopoulos and Hangarter, 1990). Lateral roots were counted under a dissecting microscope after 9 d of growth at 22°C under yellow light filters. For sucrose-dependence assays, seeds were prepared as above and plated on PN and PNS, grown 1 d under continuous white light, then wrapped with foil, and grown 5 additional days in darkness. Plants grown to maturity were transferred to soil (MetroMix 200, Scotts, Marysville, OH) after ~14 d of growth and placed under continuous white light (Sylvania Cool White fluorescent bulbs, Danvers, MA) at 22–25°C.

Mutant Rescue

The *PEX7* cDNA APZ50H10R was obtained from the Kazusa Stock Center (Asamizu *et al.*, 2000), sequence verified (Lone Star Labs, Houston, TX), and a 1.3-kb *Sma*I/*Pvu*II fragment was gel-purified and cloned into a *Sma*I-digested 35SpBARN vector (LeClere and Bartel, 2001) to generate 35S-*PEX7*. The subcloning boundaries were sequenced with vector-derived oligonucleotides to verify the insert orientation, and 35S-*PEX7* was electroporated into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986) and transformed into Col-0 and *pex7-1* plants using the floral dip method (Clough and Bent, 1998). T1 seeds were grown on PNS supplemented with 7.5 μg/ml glufonisate-ammonium (BASTA; Crescent Chemical, Augsburg, Germany); resistant plants were rescued to unsupplemented PN after 8 d and later transferred to soil. The presence of the construct in T1 plants was confirmed by PCR-amplification of genomic DNA with a vector-derived oligonucleotide and an appropriate gene-specific primer. T1 plants confirmed to carry an intact construct were grown to maturity, and T2 progeny were examined for IBA resistance.

Visualization of Matrix Protein Import Using Green Flourescent Protein

A PTS2-tagged green fluorescent protein (GFP) was created by amplifying the 5′ 147 base pairs of *Arabidopsis PED1* thiolase (Hayashi *et al.*, 1998) clone U09045 (Yamada *et al.*, 2003) that encode the N-terminal region containing the PTS2 signal sequence and the signal sequence cleavage site (Johnson and Olsen, 2003). Oligonucleotides PTS2*Xba* (5′-CCAGAAGGATCTAGAAAAAATGGAGAAAGCGATCG-3′, modifications to add a *Xba*I site and adenines 5′ of the start codon are underlined) and PTS2*Bam* (5′-CAACAGGATCCCATAGAGAGAGGTCCTCTG-3′, modifications to add a *Bam*HI site and allow in-frame subcloning are underlined) were used for PCR amplification of U09045 template DNA with *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA). The resultant 175-base pair blunt-ended product was gel-purified and subcloned into *Eco*RV-cut pBluescript KS+ (Stratagene) and sequenced to verify the absence of mutations. From this construct, the 155-base pair *Xba*I/*Bam*HI fragment was gel-purified and cloned into *Xba*I/*Bam*HI-digested CD3–326 (Davis and Vierstra, 1998) downstream of the 35S promoter and in frame with the gene encoding enhanced GFP. From this construct, a 2-kb *Eco*RI/*Hin*dIII product that included the 35S promoter and PTS2-GFP fusion was gel-purified and cloned into *Eco*RI/*Hin*dIII-digested pBIN19 plant transformation vector (Bevan, 1984) to yield PTS2-GFP. Cytoplasmic eGFP (unmodified CD3–326) and PTS2-GFP were transformed into Agrobacterium and then Col-0, *pex7-1*, and *pex5-1* plants as described above. Col-0, *pex5-1*, and *pex7-1* T1 seeds were screened on PNS with 13 μg/ml kanamycin sulfate from a water-dissolved stock, and kanamycin-resistant Col-0 and *pex5-1* T1 plants were rescued to PN plates after 9 d. The existence of a kanamycin resistance gene in the T-DNA insert in *pex7-1* T1 plants necessitated isolating transformants by screening for GFP fluorescence on a Leica MZFLIII dissecting scope (Deerfield, IL), and GFP-expressing *pex7-1* plants were rescued as above. To monitor GFP localization, T2 seedlings were mounted on slides, and GFP fluorescence in root tips of 3-d-old seedlings grown in white light suspended in 0.1% agar and root hairs of 7-d-old seedlings grown in white light on PNS were examined by using a Zeiss Axioplan 2 fluorescence microscope (Thornwood, NY) equipped with a narrow-band GFP filter set (41020, Chroma Technology, Rockingham, VT).

Col-0 plants expressing PTS1-GFP, a CD3–326 construct mutagenized to introduce the PTS1 signal sequence SKL to the extreme C-terminus of eGFP (the construct GFP-SKL from Zolman and Bartel, 2004), were crossed to *pex7-1* and *pex5-1* plants, and IBA-resistant F2 progeny were genotyped using PCR for the *pex7-1* and *pex5-1* mutations. Lines segregating GFP fluorescence were identified and used for analysis as described above.

Western Blot Analysis

Protein was extracted from entire 2-d-old Col-0, *pex7-1* and *pex5-1* seedlings grown aseptically in water under white light and 3-d-old Col-0, *pex7-1*, *pex5-1*, and *pex7-1 pex5-1* seedlings grown in 0.5% sucrose under white light. Germination is slightly delayed by sucrose, so the different chronological ages represent the equivalent developmental stages. Twenty-five seedlings with emerged embryonic roots (radicles) were selected from each genotype, frozen, ground with a pestle, and suspended in one volume of extraction buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS). Samples were briefly centrifuged to pellet debris, and supernatants were heated for 10 min at 80°C. Protein extracts were subjected to SDS-PAGE on NuPAGE 10% Bis-Tris gels (Invitro-

gen, Carlsbad, CA) with Cruz size standards (Santa Cruz Biotechnology, Santa Cruz, CA).

Protein was transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at 24 V. Membranes were blocked in 5% powdered milk in Tween Tris-buffered saline (TTBS) buffer (Ausubel *et al.,* 1995) for at least 2 h, and then incubated with rocking at 4°C for at least 13 h in a 1:1000 dilution of rabbit antiplant thiolase antibody (Kato *et al.,* 1996) or a 1:200 dilution of a rabbit anti-*Arabidopsis* PEX5 antibody (Zolman and Bartel, 2004) in 5% milk TTBS. Membranes were washed four times in 5% milk TTBS and then incubated for at least 1 h at room temperature in a 1:500 dilution of a horseradish peroxidase–conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). Membranes were washed as before and visualized using LumiGLO reagent (Cell Signaling, Beverly, MA).

PEX7 Expression Analysis

Seven-day-old Col-0, *pex7-1*, *pex5-1*, and *pex7-1 pex5-1* seedlings grown under white light on PNS were frozen in liquid nitrogen and ground in a chilled mortar and pestle. RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Valencia, CA). After OD_{260} quantification and electrophoresis to ensure the absence of RNA degradation, each sample was treated with DNaseI (Amplification Grade, Roche Applied Science, Indianapolis, IN) and 0.3 μg was reverse-transcribed in a 20 μL volume using random hexamer primers and SuperScript III polymerase (Invitrogen). Controls lacking reverse transcriptase were performed for each sample.

Quantitative real-time reverse-transcription PCR using an ABI Prism 7000 Sequence Detection System was performed on triplicate 25-μL reactions containing 2 μL cDNA, 0.5 μM each primer, and 0.2 μM probe in Taqman universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers for *PEX7* were PEX7-QRTF (5′-TGGCTGTGCTTAATGGTCATG-3′) and PEX7-QRTR (5′-CCTCCTATGCGGCGAGAA-3′) and a 5′ 6-FAM–labeled, 3′ MGBNFQ (minor groove-binder/nonfluorescent quencher) probe (5′-ATATGCGGTGAGGAAGG-3′). Primers for the *APRT* control were APRT-F (5′-CATATCTGTTGTTGCAGGTGTTGA-3′) and APRT-R (5′-CCAATAGCCAACGCAATAGGA-3′) and a 5′ 6-FAM–labeled, 3′ MGBNFQ-quenched probe (5′-TAGAGGTTTCATTTTTGGCC-3′). *APRT* amplification was used to normalize *PEX7* levels using a comparative C_T method (ABI Prism 7700 Sequence Detection System User Bulletin no. 2, http://www.appliedbiosystems.com). Control reactions without reverse transcriptase amplified later than experimental samples.

RESULTS

Peroxisome Defects in the Arabidopsis pex7-1 Mutant

Sequence similarity searches have been used to identify *At1g29260* as *Arabidopsis PEX7* (Schumann *et al.,* 1999). The single-copy gene encodes a protein ~40 and ~59% identical to PEX7 from *S. cerevisiae* and humans, respectively, and is largely comprised of WD40 domains (Figure 1, A and B). To determine the role of PEX7 in plant development, we isolated the *pex7-1* mutant from the Salk Institute T-DNA insertion collection (Alonso *et al.,* 2003) and identified a single kanamycin resistance–linked T-DNA 95 base pairs upstream of the start codon, placing it within the 5′ untranslated region.

Like previously described *Arabidopsis* mutants with peroxisomal defects (Zolman *et al.,* 2000, 2001a, 2001b; Zolman and Bartel, 2004), *pex7-1* displays reduced sensitivity to exogenously supplied IBA (Figure 2A). In addition, the mutant is resistant to an analogous synthetic proto-auxin, 2,4-dichlorophenoxybutyric acid (2,4-DB; unpublished data), which is converted in peroxisomes to the active derivative 2,4-dichlorophenoxyacetic acid (2,4-D; Hayashi *et al.,* 1998). The IBA resistance of *pex7-1* is rescued by overexpression of wild-type *PEX7* (Figure 2B), indicating that the phenotype observed results from reduced PEX7 function.

Some peroxisomal mutants produce fewer lateral roots than wild type even without exogenous auxin (Zolman *et al.,* 2001b; Zolman and Bartel, 2004). Any lateral root production defect in *pex7-1* on unsupplemented medium is weak (Figure 2C), though reproducible. In addition, *pex7-1* forms fewer lateral roots than wild type after IBA treatment (unpublished data). *pex7-1* is not generally defective in auxin responses, because it responds normally to IAA both in primary root elongation inhibition and induction of lateral roots (unpublished data). Whereas defects in β-oxidation of fatty acids stored in seeds renders many peroxisome-defective mutants completely sucrose dependent for seedling establishment (Hayashi *et al.,* 1998, 2000; Zolman *et al.,* 2001a, 2001b; Footitt *et al.,* 2002; Fulda *et al.,* 2004; Zolman and Bartel, 2004), the *pex7-1* hypocotyl is not markedly shorter than wild type when grown in darkness without added sucrose (Figure 2D). This lack of sucrose dependence suggests that any defect in peroxisomal β-oxidation of endogenous fatty acids in the *pex7-1* mutant may be modest. Adult *pex7-1* plants are not discernibly different from wild type in growth rate or morphology (unpublished data).

To test whether matrix proteins are imported normally into *pex7-1* peroxisomes, we expressed PTS1- and PTS2-tagged versions of GFP in the *pex7-1* mutant. As expected, PTS1-tagged GFP (Zolman and Bartel, 2004) is efficiently imported into peroxisomes in *pex7-1* (Figure 3). The presence of PTS1-GFP in a normal punctate pattern in *pex7-1* roots indicates that peroxisome abundance and gross morphology are unaffected by PEX7 deficiency. In contrast, PTS2-tagged GFP is not efficiently imported into *pex7-1* peroxisomes; although some faint punctate fluorescence is still observed, PTS2-GFP confers largely diffuse fluorescence similar to cytoplasmically localized GFP (Figure 3). To confirm that this defect observed with an engineered substrate reflected endogenous proteins, we used Western blotting to indirectly examine import of 3-ketoacyl-CoA thiolase (thiolase) into peroxisomes. As the PTS2 signal is removed from thiolase following import, the consequent molecular weight shift is diagnostic of peroxisomal import. We detected primarily mature processed thiolase in wild-type seedlings, but some residual unprocessed thiolase remains in *pex7-1* seedlings (Figure 4A), especially in seedlings grown in the absence of sucrose (Figure 4B). These results imply that peroxisomal deficiencies observed in *pex7-1* result from poor import of PTS2-containing proteins.

PTS2-containing Protein Import Is Defective in the PTS1 Receptor Mutant pex5-1

Arabidopsis contains a single *PEX5* gene (*At5g56290*; Brickner *et al.,* 1998; Zolman *et al.,* 2000) encoding a protein ~20 and ~28% identical to *S. cerevisiae* Pex5p and human PEX5L, respectively (Figure 1). PEX5 proteins contain N-terminal pentapeptide repeat (PPR) domains involved in PEX14 docking at the peroxisome (Nito *et al.,* 2002) and C-terminal tetratricopeptide repeat (TPR) domains necessary for PTS1 protein cargo binding (Gatto *et al.,* 2000). In addition, among the TPR repeats is a sequence conserved between human and plant PEX5 proteins and the PEX7-binding domains of the yeast peroxisome docking proteins Pex18p and Pex21p (Einwächter *et al.,* 2001). A fragment of human PEX5L including this region is sufficient for interaction with human PEX7 (Dodt *et al.,* 2001).

The previously described *pex5-1* mutant displays reduced sensitivity to exogenous IBA and a slight growth defect in darkness without exogenous sucrose (Zolman *et al.,* 2000). *pex5-1* contains a serine to leucine mutation in the presumptive PEX7-binding region (Figure 1). Western blot analysis indicates that the pex5-1 mutant protein accumulates to wild-type levels (Figure 4C). Intriguingly, the analogous serine is mutated to phenylalanine in a CHO cell line defective in PTS2, but not PTS1, protein import (Matsumura *et al.,* 2000), suggesting that the *Arabidopsis pex5-1* mutant might similarly have defects in PTS2 import.

To directly examine PTS1 and PTS2 function in the *pex5-1* mutant, we observed localization of PTS1- and PTS2-tar-

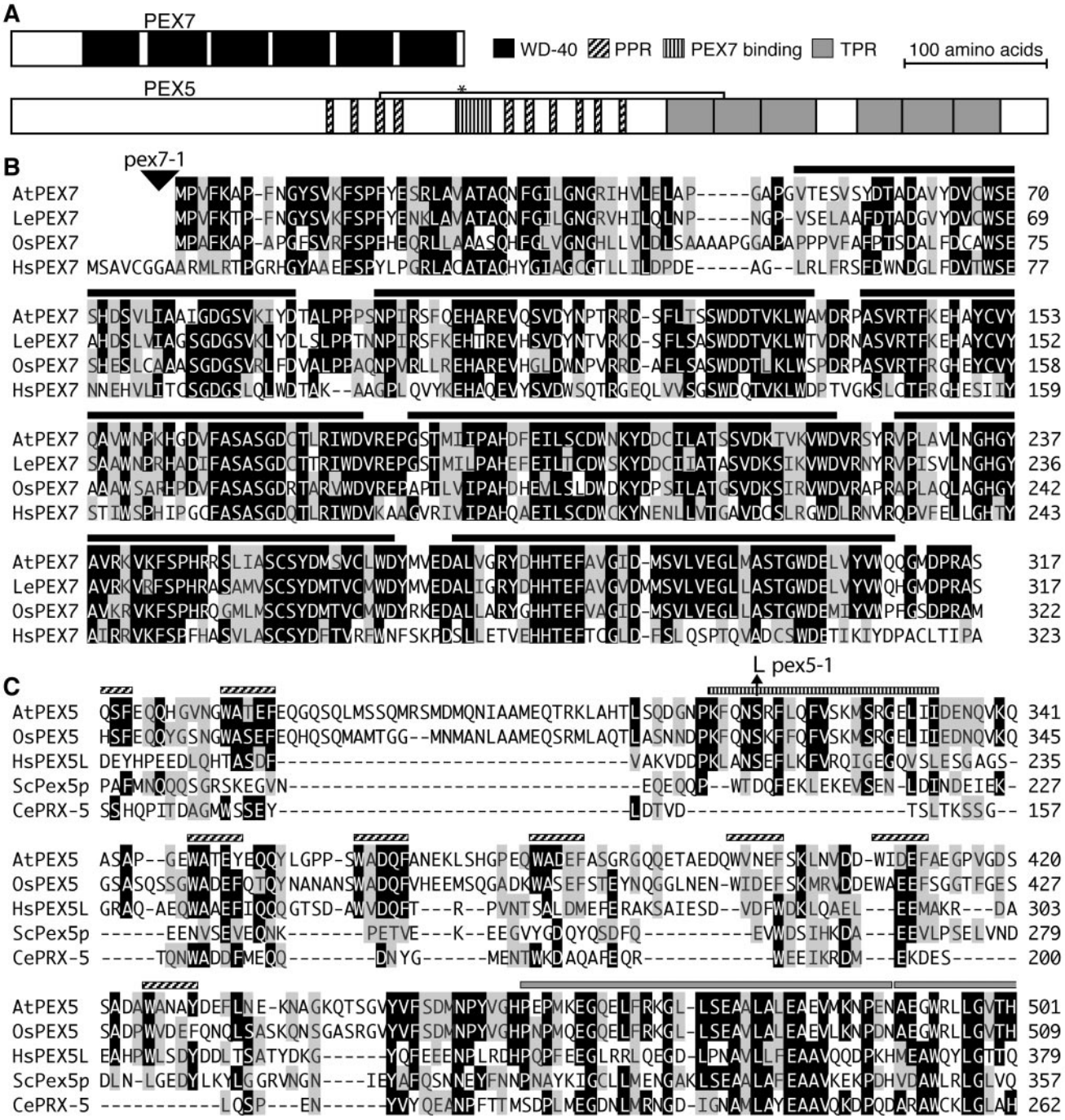

Figure 1. Organization of *Arabidopsis* PEX7 and PEX5 proteins. (A) Schematic showing domain architecture of *Arabidopsis* PEX7 and PEX5 proteins. The bracket above PEX5 is the region shown in C, and the asterisk indicates the Ser to Leu mutation indicated in C. (B) PEX7 is the single *Arabidopsis* (At) homolog of human (Hs) PEX7 and is highly similar to tomato (Le) GenBank accession AY186749 and rice (Os) TIGR gene temporary ID 8351.t01315 (OsPEX7). (C) Partial alignment of *Arabidopsis* PEX5 with orthologues from rice, human, yeast (Sc), and roundworm (Ce). *pex5-1* contains a missense mutation in a conserved serine (Zolman *et al.,* 2000) needed for PEX7 interaction and PTS2-containing protein import into peroxisomes in Chinese hamster (Matsumura *et al.,* 2000). Note the absence of this domain in CePEX5; *C. elegans* does not contain *PEX7* or a PTS2 import pathway (Gurvitz *et al.,* 2000; Motley *et al.,* 2000). Sequences were aligned with the MegAlign program (DNA Star, Madison, WI) using the ClustalW method; residues identical in three or more sequences are shaded black, residues chemically similar in three or more sequences are shaded gray. WD-40 domains determined by Pfam (Bateman *et al.,* 2002); PPR and TPR domains after Zolman *et al.* (2000).

geted GFP derivatives. Interestingly, we found that *pex5-1* is fully competent in PTS1-GFP import (Figure 3). In marked contrast, however, PTS2-GFP is not detectably imported into peroxisomes in *pex5-1* (Figure 3), consistent with the hypothesis that the primary defect in the *pex5-1* mutant is in PTS2, rather than PTS1, import. Indeed, PTS2-targeted thiolase is imported less efficiently in *pex5-1* than in wild type or *pex7-1* (Figure 4, A and B).

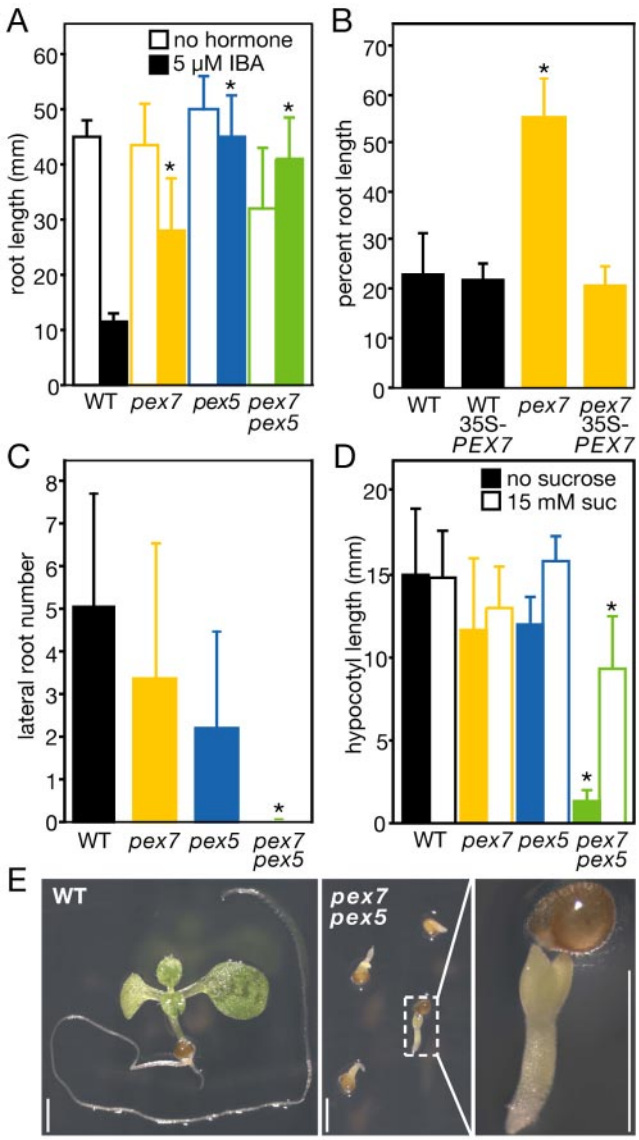

Figure 2. *pex7-1*, *pex5-1*, and *pex7-1 pex5-1* are deficient in peroxisomal processes. (A) Root elongation on IBA. Root lengths of 8-d-old plants grown on sucrose-supplemented medium with or without addition of 5 μM IBA under yellow light filters are shown. (B) Percent root elongation on 5 μM IBA versus hormone-free medium. Plants were grown as in A. (C) Lateral root number. Lateral roots of 9-d-old plants grown on PNS under yellow light filters were counted under a dissecting microscope. (D) Hypocotyl elongation in darkness with and without sucrose. Plants were grown one day in white light and then were grown in darkness for 5 additional days. Bars, means + SDs; $n \geq 6$ in A, $n \geq 10$ in B–D. *Significantly different from wild type ($p < 0.01$; one-tailed t test assuming unequal variance). (E) *pex7-1 pex5-1* does not develop after germination without sucrose in the light. Wild-type (left) or *pex7-1 pex5-1* (right) plants were grown for 7 d under white light on medium lacking sucrose. Scale bars, 1 mm.

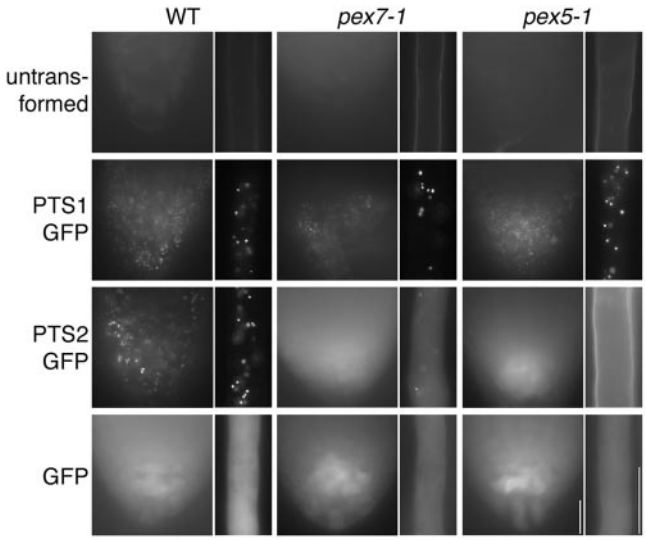

Figure 3. *pex7-1* and *pex5-1* are deficient in PTS2 protein import into peroxisomes. Three-day-old root tips from seedlings grown in 0.1% agar in white light (left panels) and 7-d-old root hair cells (right panels) are shown for wild-type, *pex7-1*, and *pex5-1*, and transformants expressing PTS1-GFP, PTS2-GFP, and cytoplasmic GFP. Note the punctate fluorescence in plants expressing PTS1-GFP and wild-type plants expressing PTS2-GFP; fluorescence is diffuse in *pex7-1* and *pex5-1* expressing PTS2-GFP, indicating disrupted PTS2 protein import into peroxisomes. Untransformed plant images were captured at the maximum exposure time used for any of the transformed lines. Scale bars, 200 μm.

Severe Developmental Deficiencies and Blocked PTS2-Protein Import in pex7-1 pex5-1

pex7-1 and *pex5-1* were cross-pollinated to produce a *pex7-1 pex5-1* double mutant. Like both parents, the double mutant displays reduced sensitivity to exogenous IBA (Figure 2A). Unlike either single mutant, however, *pex7-1 pex5-1* is completely dependent on exogenous sucrose for growth not only in darkness (Figure 2D), but also in light (Figure 2E). Even when provided with sucrose, the double mutant grows more slowly than wild type, as evidenced by reduced root and hypocotyl elongation (Figure 2, A and D). Moreover, the possible lateral root defects observed in the single mutants are exacerbated in the double mutant; 9-d-old *pex7-1 pex5-1* seedlings lack lateral roots (Figure 2C). Consistent with these morphological defects, 3-d-old double mutant seedlings apparently lack processed thiolase (Figure 4A), suggesting a severe block in import of thiolase, and presumably other PTS2 proteins, into peroxisomes.

In addition to these seedling defects, adult *pex7-1 pex5-1* plants are dramatically less robust and less fecund than wild type or either parent (Figure 5, C and D). Examination of siliques of different ages reveals apparently normal fertilization indicated by the presence of plump developing seeds in green siliques, but defects become increasingly apparent during the course of seed maturation (Figure 5A). A majority of mature double mutant seeds are shrunken (Figure 5, B and D). Seed development is dependent upon *PEX7* dosage; plants homozygous for *pex5-1* and heterozygous for *pex7-1* generate an intermediate number of shrunken seeds (Figure 5D). In contrast, *pex5-1* is fully recessive for seed morphology in the *pex7-1* background, as *pex7-1/pex7-1 PEX5/pex5-1* plants produce normal seeds (Figure 5D). Among *pex7-1 pex5-1* seeds with a wild-type appearance, fewer than half germinate when grown on media supplemented with sucrose, whereas only occasional abnormally shaped seeds germinate (Figure 5E).

Among *pex7-1 pex5-1* mutant seeds that germinate, abnormalities in embryonic development become apparent (Figure 6). About 15% of mutant seedlings exhibit various degrees of cotyledon fusion (Figure 6), whereas such aberrant seedling morphology is seen in fewer than 1.2% of wild type

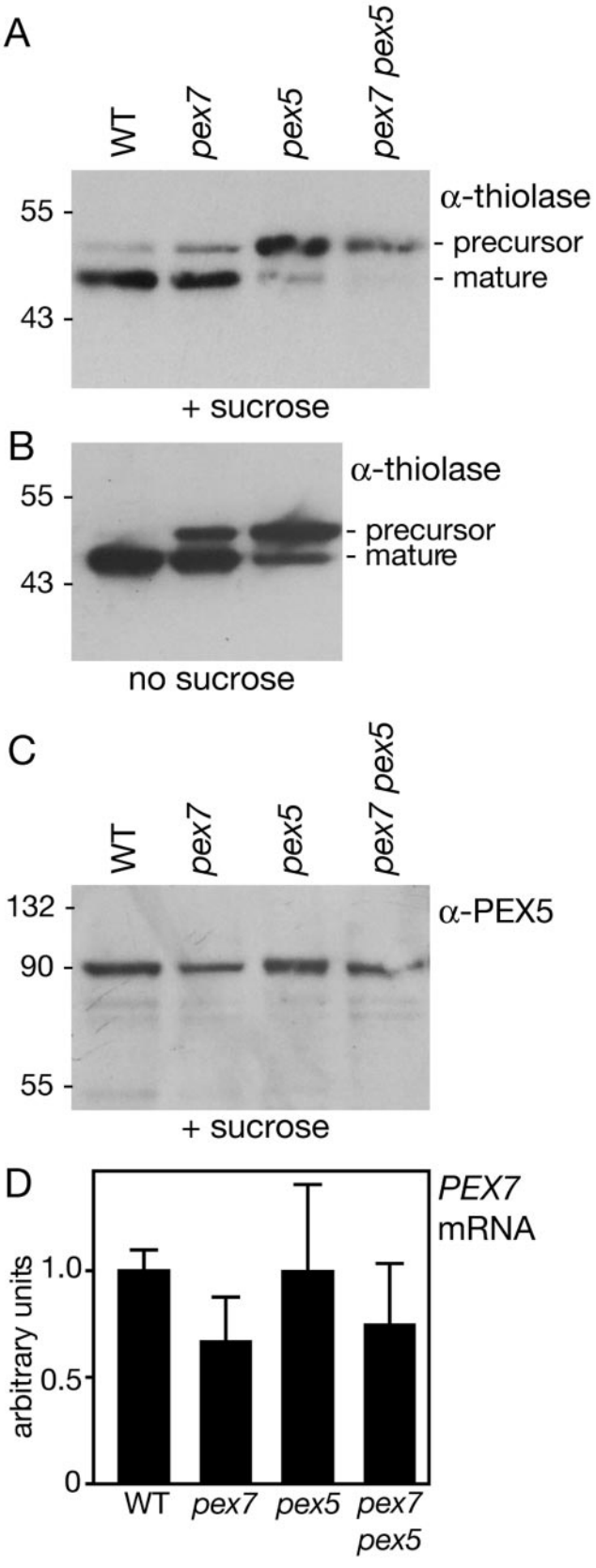

Figure 4. *pex7-1* and *pex5-1* are deficient in import of the PTS2 protein thiolase into peroxisomes. (A) Thiolase import defects. Precursor thiolase protein is translated with an N-terminal, PTS2-containing sequence that is cleaved after entry into peroxisomes to produce mature thiolase. Protein was extracted from seedlings grown on sucrose and visualized using an antithiolase antibody (Kato *et al.*, 1996). (B) Thiolase import defects are exaggerated in plants grown without exogenous sucrose. *pex7-1 pex5-1* was omitted because of developmental arrest in the absence of sucrose. (C) PEX5 protein is present in all mutants. Protein was extracted from seedlings grown on sucrose and visualized using anti-PEX5 antibody (Zolman and Bartel, 2004). Positions of molecular mass markers (in kDa) are indicated at the left in A–C. (D) *PEX7* message is present in all mutants. *PEX7* mRNA levels relative to an *APRT* control in wild type and each mutant were determined using quantitative real-time reverse-transcription PCR. Error bars, SDs of mean *PEX7* levels expressed in arbitrary units.

or either single mutant ($n \geq 112$, unpublished data). Most *pex7-1 pex5-1* seedlings with fused cotyledons develop into adult plants resembling siblings with unfused cotyledons, though a small percentage arrest and perish as seedlings (unpublished data).

DISCUSSION

Using a reverse-genetic approach, we demonstrated that *Arabidopsis PEX7* is necessary for PTS2 protein import into peroxisomes and that plant PEX7 function is relevant in vivo. The *pex7-1* mutant is resistant to the proto-auxin IBA (Figure 2A), which is converted in peroxisomes into the active auxin IAA (Zolman *et al.*, 2000). This phenotype was rescued by 35S promoter-driven overexpression of *PEX7*. The *pex7-1* mutant is not notably impaired when grown without light and sucrose, suggesting that the endogenous fatty acid β-oxidation needed for seedling development in these conditions is minimally affected. The relatively weak *pex7-1* phenotype may be attributable to the nature of the lesion; the T-DNA insertion in *pex7-1*, though within the 5′ UTR of a cDNA (Schmid *et al.*, 2003), allows some expression of presumably fully functional PEX7 (Figure 4D). When we examined PTS2-GFP localization in *pex7-1*, we found little punctate fluorescence (Figure 3), indicating a severe block in PTS2 protein import. However, the apparent severity of *pex7-1* in this assay could result from flooding the cells with 35S-expressed PTS2-GFP. Indeed, the endogenous PTS2-targeted thiolase import is only slightly affected in *pex7-1* (Figure 4, A and B), revealing the sensitivity of IBA resistance and PTS2-GFP localization assays in detecting peroxisome defects.

Because PEX7 appears to deliver cargo to the peroxisome via interaction with PEX5 in *Arabidopsis* (Nito *et al.*, 2002; Sparkes and Baker, 2002; Johnson and Olsen, 2003) as it does in mammals (Braverman *et al.*, 1998; Matsumura *et al.*, 2000; Dodt *et al.*, 2001), we also examined PTS1- and PTS2-protein import in the existing *pex5-1* allele (Zolman *et al.*, 2000). Interestingly, PTS1-GFP localization was properly punctate in *pex5-1*, whereas PTS2-GFP was diffuse (Figure 3). Moreover, PTS2-containing thiolase was inefficiently imported into *pex5-1* peroxisomes (Figure 4, A and B). There is one existing *pex5* mutant of this type, a CHO cell line with a PTS2-specific defect resulting from the inability of PEX7 to bind mutant pex5 (Matsumura *et al.*, 2000). Remarkably, the lesion in the CHO mutant pex5 is an analogous serine to that mutated in pex5-1 (Figure 1). Moreover, both are mutated to hydrophobic amino acids, leucine in pex5-1 and phenylalanine in the CHO mutant. The characterization of these two *pex5* alleles with PTS2-specific defects demonstrates the critical importance of this conserved serine for PEX7 binding and suggests an ancient origin for PEX7-PEX5 interaction.

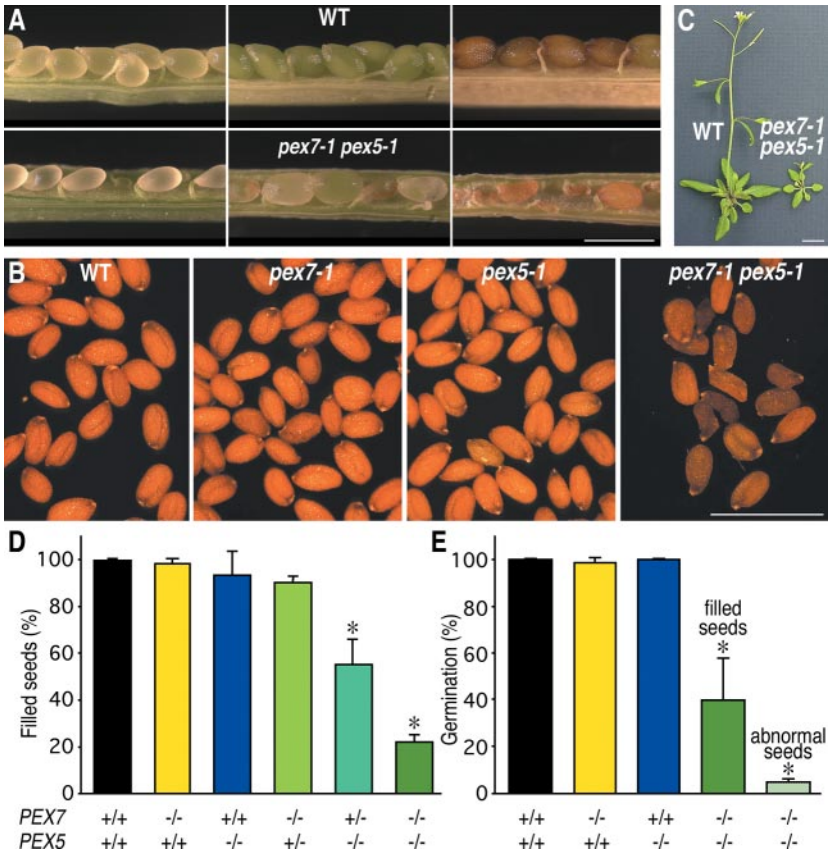

Figure 5. *pex7-1 pex5-1* double mutant phenotypes. (A) Seed development is aberrant in *pex7-1 pex5-1*. One valve was removed from wild-type and *pex7-1 pex5-1* siliques to reveal developing seeds of increasing age from left to right. Scale bar, 1 mm. (B) Mature *pex7-1 pex5-1* seeds are shrunken. Scale bar, 1 mm. (C) *pex7-1 pex5-1* adult plants have reduced stature. Plants were grown under white light on PNS for 14 d, then transferred to soil, and grown an additional 17 d. Scale bar, 1 cm. (D) Percent seeds with normal filled morphology. Mature seeds were assayed for plump appearance. Bars, means + SDs from progeny of three plants of the indicated genotype; $n \geq 60$ seeds per plant. (E) Percent germination in single and double mutants. *pex7-1 pex5-1* were sorted by seed morphology in D. Seedlings were grown on medium supplemented with 45 mM sucrose and assayed for germination (radicle emergence) after 9 d. Bars, means + SDs (or SE of measurement for *pex5-1*) from progeny of three plants of the indicated genotype (progeny of two *pex5-1* plants); $n \geq 23$ seeds per plant, except *pex7-1 pex5-1* filled seeds where $n \geq 10$ seeds per plant. *Significantly different from wild type ($p < 0.02$; one-tailed *t* test assuming unequal variance).

Further, whereas the CHO cell line was isolated in a screen that selected for PTS2-deficient import (Matsumura *et al.*, 2000), *Arabidopsis pex5-1* was isolated from a screen for IBA resistance, which can yield a variety of peroxisomal defects (Zolman *et al.*, 2000, 2001a, 2001b; Zolman and Bartel, 2004); this result may suggest that PTS2 proteins are particularly important for IBA metabolism in seedlings. Indeed, the peroxisome-defective mutant *ped2/pex14* is deficient in IBA (Monroe-Augustus, 2004) and 2,4-DB (Hayashi *et al.*, 2000) response as well as PTS2-protein import (Hayashi *et al.*, 2000).

Though an N-terminal *Arabidopsis* PEX5 fragment lacking the region containing the *pex5-1* mutation has been shown to interact with PEX7 in a yeast two-hybrid assay, the interaction was less robust than with full-length PEX5 (Nito *et al.*, 2002). Further, the interaction was enhanced in a PEX5 fragment containing the region mutated in *pex5-1* (Nito *et al.*, 2002). Thus, the region we implicate in PTS2 import is necessary, but may not be sufficient, for the PEX5-PEX7 interaction driving PTS2 import in vivo.

Whereas both *pex7-1* and *pex5-1* single mutants are peroxisomally deficient in only the most sensitive assays, combining the two defects yields a severely affected double mutant with numerous developmental abnormalities, likely resulting from reduced *PEX7* expression combined with inefficient PEX7-PEX5 interaction (Figure 7). PTS2-targeted thiolase import is severely affected in *pex7-1 pex5-1*, whereas *PEX7* expression and PEX5 levels are similar to those in the respective single mutants (Figure 4); this result is consistent with the double mutant harboring two partial loss-of-function mutations in the same pathway. *pex7-1 pex5-1* requires exogenous sucrose for seedling establishment (Figure 2E), but not for growth as an adult plant. The sucrose dependence of this mutant demonstrates the necessity of PTS2 protein import for utilization of seed-storage lipids by seedlings before photosynthesis is established, a process of specialized peroxisomes termed glyoxysomes (Beevers, 2002).

Though the PTS2 signal sequence is less common than PTS1, it is more common in plants than other organisms

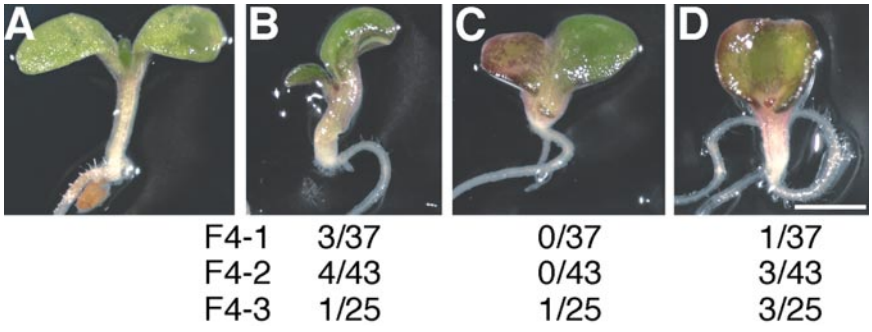

Figure 6. Cotyledon fusion in the *pex7-1 pex5-1* double mutant. *pex7-1 pex5-1* plants were grown for 7 d on sucrose-supplemented medium and frequencies of plants with (A) wild-type, (B) asymmetric, (C) partially fused, and (D) fused cotyledons were determined. Ratios below B, C, and D represent fraction of *pex7-1 pex5-1* with the indicated degree of fusion among progeny of three double mutant plants. Scale bar, 1 mm.

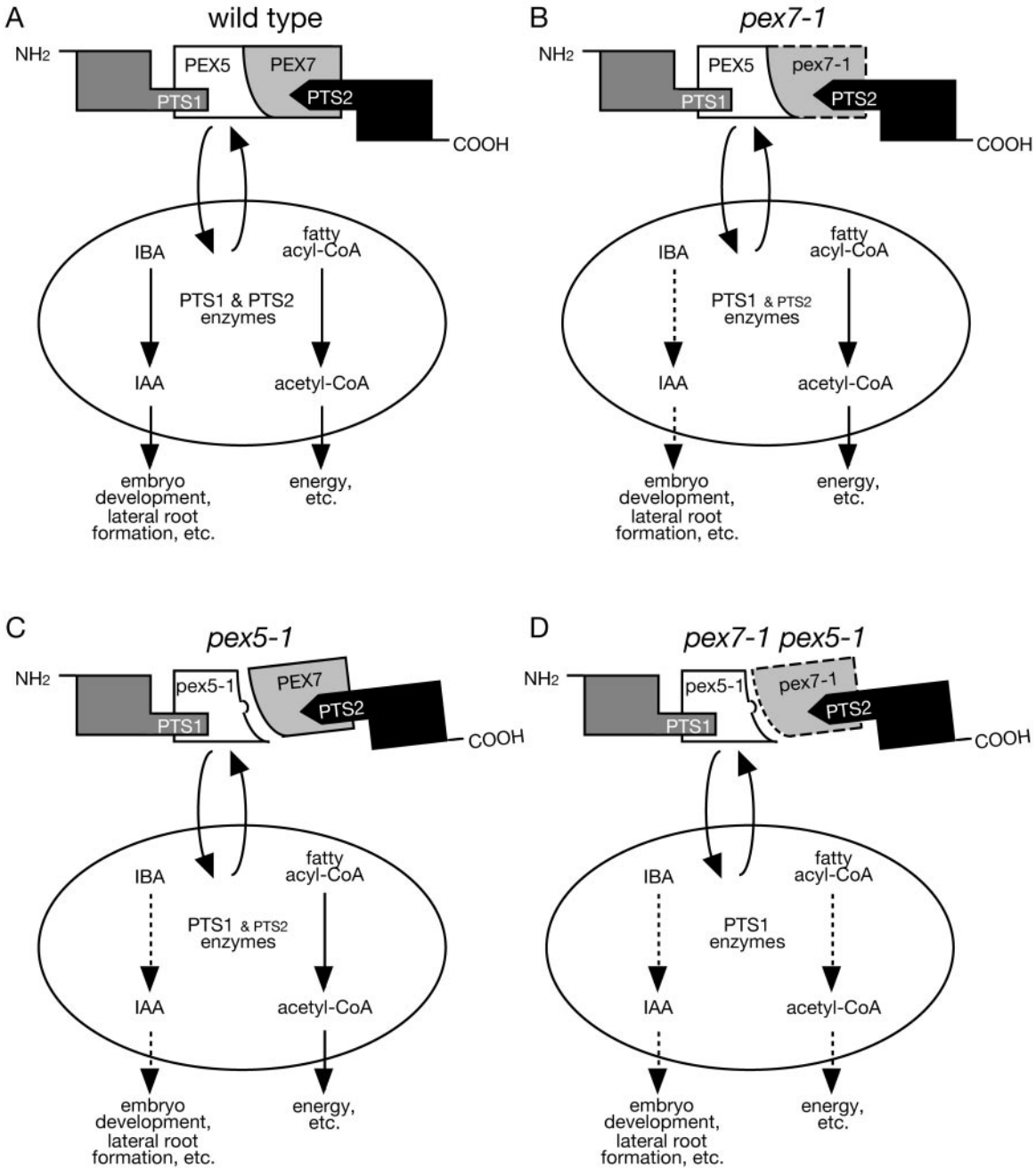

Figure 7. Model for PTS2 protein import into plant peroxisomes. (A) PEX7 binds both PTS2 protein cargo and the PTS1 protein receptor PEX5. Delivery of PTS2 protein into the peroxisome is dependent on complex formation with PEX5. PTS2 protein cargo is necessary for peroxisomal processes of fatty acid β-oxidation and conversion of IBA into the active auxin IAA. (B) Functional PEX7 may be expressed at reduced levels in the *pex7-1* mutant, resulting in reduced PTS2 protein import and IBA resistance. (C) PEX7 binding with pex5-1 is inefficient, resulting in decreased PTS2 protein import and IBA resistance. (D) Two partial defects are combined in *pex7-1 pex5-1* to nearly eliminate PTS2 protein import, resulting in severe developmental defects.

(Johnson and Olsen, 2001; Mullen, 2002) and is greatly overrepresented among β-oxidation enzymes highly expressed during the glyoxysome-dependent seedling establishment phase (Kamada *et al.,* 2003). PTS2-bearing β-oxidation enzymes with peak expression in seedlings (Kamada *et al.,* 2003) include the acyl-CoA synthases LACS6 and LACS7 (Fulda *et al.,* 2002), the acyl-CoA oxidases ACX2 (Hooks *et al.,* 1999) and ACX3 (Froman *et al.,* 2000), and the thiolase PED1/KAT2 (Hayashi *et al.,* 1998; Germain *et al.,* 2001). Among these enzymes, *ped1/kat2* mutants (Hayashi *et al.,* 1998; Germain *et al.,* 2001) and the *lacs6 lacs7* double mutant require exogenous sucrose for seedling establishment (Fulda *et al.,* 2004). Although both LACS6 and LACS7 have functional PTS2 signal sequences, LACS7 also possesses a functional PTS1 sequence, and the *lacs6* single mutant is sucrose-independent (Fulda *et al.,* 2002), making these proteins unlikely causes of sucrose dependence in the PTS2-import deficient *pex7-1 pex5-1*. However, sucrose dependence in

pex7-1 pex5-1 could result from mislocalization of the PTS2-targeted PED1/KAT2 to the cytosol (Figure 4A). In contrast to these β-oxidation enzymes, all known enzymes directly involved in photorespiration in green leaf peroxisomes bear PTS1 signal sequences (Reumann, 2002); the PTS2-independence of photorespiration could account for the healthy green coloration of the *pex7-1 pex5-1* mutant (Figure 5C), which is unlike the pale-colored *pex14* and *pex6* mutants (Hayashi *et al.*, 2000; Zolman and Bartel, 2004).

pex7-1 pex5-1 plants germinated on sucrose and transferred to soil grow into stunted adults that produce few normal seeds, only a minority of which germinate, even when supplied with sucrose (Figure 5). Thus, PTS2 import is required not only for seedling establishment, but also for vegetative growth and normal seed development. Strikingly, the *sse1* mutant, which is defective in PEX16, a peroxin implicated in early steps in peroxisome formation, was isolated because it bears ~90% inviable shrunken seeds (Lin *et al.*, 1999). Further, null mutations in *PEX2* and *PEX10* are lethal at stages before seed maturation (Hu *et al.*, 2002; Schumann *et al.*, 2003; Sparkes *et al.*, 2003). Thus, the high frequency of shrunken and inviable seeds in the *pex7-1 pex5-1* double mutant again implicates peroxisomes in proper seed development and reveals the necessity of PTS2 protein import for this process.

Those *pex7-1 pex5-1* seeds that do germinate often display fused cotyledons (Figure 6). Several *Arabidopsis* mutants with defective cotyledon separation have been described, and these link cotyledon development with auxin response. The *pin-formed1* mutant has frequent cotyledon fusion and is defective in an auxin efflux facilitator that is necessary to establish auxin gradients in developing embryos (Gälweiler *et al.*, 1998). The *monopteros* mutant likewise has variably fused cotyledons and is defective in an auxin response factor that interprets auxin gradients (Hardtke and Berleth, 1998). The *cup-shaped cotyledon* double mutant *cuc1 cuc2* was isolated on the basis of cotyledon fusion and is defective in functionally redundant transcription factor genes (Aida *et al.*, 1997; Takada *et al.*, 2001) that are misregulated in *monopteros* and *pin-formed1* (Aida *et al.*, 2002). Thus, auxin signaling is critical for proper cotyledon development.

The requirement of auxin response for proper embryonic symmetry, the requirement for peroxisome function to allow IBA conversion into active IAA, and the reduced auxin response phenotype of *pex7-1 pex5-1* implicate IBA as a critical auxin reservoir during embryogenesis. Though IBA levels have not been quantified in seeds, IBA is present in seedling tissue at nearly the levels of free IAA (Ludwig-Müller *et al.*, 1993).

PEX7 is necessary for peroxisome function in mammals and yeast (Rehling *et al.*, 1996; Mukai *et al.*, 2002), and characterization of the first plant *pex7* mutant reveals a reduction in PTS2 protein import into peroxisomes. We also show that the sole described plant *pex5* mutant is defective in PTS2-, but not PTS1-protein import. *pex7-1 pex5-1* double mutants have severe PTS2 import defects, several developmental abnormalities, and a high frequency of embryonic death and deformities, some of which may result from defective IBA metabolism. It will be interesting to observe the phenotypes of hypothetical *pex5* mutants specifically defective in PTS1 rather than PTS2 import, which will reveal the roles for PTS1 import in plant development. In addition, the functional necessity of PEX7 interaction with PEX5 observed previously only in mammals further establishes *Arabidopsis* as an excellent model for human peroxisome biogenesis disorders.

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