Disparate peroxisome-related defects in *Arabidopsis pex6* and *pex26* mutants link peroxisomal retrotranslocation and oil body utilization

Kim L. Gonzalez, Wendell A. Fleming, Yun-Ting Kao, Zachary J. Wright, Savina V. Venkova, Meredith J. Ventura†, and Bonnie Bartel*†

Department of Biosciences, Rice University, Houston TX USA

**SUMMARY**

Catabolism of fatty acids stored in oil bodies is essential for *Arabidopsis* seed germination and seedling development. This fatty acid breakdown occurs in peroxisomes, organelles that sequester oxidative reactions. Import of peroxisomal enzymes is facilitated by peroxins including PEX5, a receptor that delivers cargo proteins from the cytosol to the peroxisomal matrix. After cargo delivery, a complex of the PEX1 and PEX6 ATPases and the PEX26 tail-anchored membrane protein removes ubiquitinated PEX5 from the peroxisomal membrane. We identified *Arabidopsis pex6* and *pex26* mutants by screening for inefficient seedling $\beta$-oxidation phenotypes. The mutants displayed distinct defects in growth, response to a peroxisomally metabolized auxin precursor, and peroxisomal protein import. The low PEX5 levels in these mutants were increased by proteasome inhibitor treatment or by combining *pex26* with peroxisome-associated ubiquitination machinery mutants, suggesting that ubiquitinated PEX5 is degraded by the proteasome when PEX6 or PEX26 function is reduced. Combining *pex26* with mutations that increase PEX5 levels either worsened or improved *pex26* physiological and molecular defects, depending on the introduced lesion. Moreover, elevating PEX5 levels via a 35S:PEX5 transgene exacerbated *pex26* defects and ameliorated the defects of only a subset of *pex6* alleles, implying that decreased PEX5 is not the sole molecular deficiency in these mutants. We found peroxisomes clustered around persisting oil bodies in *pex6* and *pex26* seedlings, suggesting a role for peroxisomal retrotranslocation machinery in oil body utilization. The disparate phenotypes of these *pex* alleles may reflect unanticipated functions of the peroxisomal ATPase complex.

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*Corresponding author: Bonnie Bartel, Department of Biosciences, MS-140, Rice University, 6100 Main St., Houston TX, USA. Phone: 713-348-5602, Fax: 713-348-5154; bartel@rice.edu.

†Present address: School of Medicine, Baylor College of Medicine, Houston, Texas, 77030

**ACCESSION NUMBERS**

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *PEX6* (At1g03000), *PEX26* (At3g10572). *PEX6* homologs in Figure 1e were *A. thaliana* PEX6 (NP_171799.2) and PEX1 (NP_196464) and *Homo sapiens* PEX6 (NP_004095), PEX1 (NP_004094), and p97 (NP_009057). PEX26 homologs in Figure 2e were from *A. thaliana* (NP_50555.1), *A. lyrata* (XP_002883823.1), *Populus trichocarpa* (XP_002309027.1), *Oryza sativa* (NP_001058494.1), *Brachypodium distachyon* (XP_003563521.1), and *Picea sitchensis* (AED77879.1). The p97 coordinates used to generate Figure 1f were from PDB ID 5C18.

ALL CO-AUTHORS CONFIRM THEY HAVE NO CONFLICTS OF INTEREST TO DECLARE
**INTRODUCTION**

Catabolism of stored fixed carbon provides seedlings with energy for germination and development prior to the onset of photosynthesis. In *Arabidopsis thaliana* seeds, this carbon is predominantly stored as triacylglycerol (TAG) in oil bodies. During germination, peroxisomes congregate with oil bodies (Chapman and Trelease, 1991; Hayashi *et al.*, 2001) to facilitate TAG utilization. The SUGAR-DEPENDENT1 (SDP1) peroxisome-associated lipase docks at oil bodies (Eastmond, 2006; Thazar-Poulot *et al.*, 2015) and releases free fatty acids from TAG (Eastmond, 2006). Free fatty acids are activated with CoA and imported into the peroxisome (Nyathi *et al.*, 2010), where \(\beta\)-oxidation of fatty acyl-CoA to acetyl-CoA contributes to sucrose production (reviewed in Graham, 2008). Because peroxisomes are the sole site of fatty acid \(\beta\)-oxidation in plants (reviewed in Graham, 2008), peroxisome dysfunction confers seedling growth defects; these defects can be alleviated by providing a fixed carbon source, such as sucrose, in the medium (reviewed in Bartel *et al.*, 2014). Plant peroxisomes also house \(\beta\)-oxidation of the hormone precursor indole-3-butyric acid (IBA) to the active auxin indole-3-acetic acid (IAA) (Zolman *et al.*, 2000; Strader *et al.*, 2010). The discovery that dysfunctional peroxisomes impair seedling IBA responses has enabled forward-genetic screens for peroxisome-defective mutants in plants (reviewed in Bartel *et al.*, 2014).

Peroxisomes are assembled and maintained through the action of peroxin (PEX) proteins. Peroxisomal matrix proteins carry peroxisomal targeting signals (PTS) that facilitate post-translational import from the cytosol via the import peroxins (reviewed in Hu *et al.*, 2012). The PTS1 is a C-terminal three-amino acid (aa) sequence (reviewed in Reumann *et al.*, 2016). The PTS2 is a nine-aa sequence within an approximately 30-aa N-terminal region that is removed after import in plants (Helm *et al.*, 2007; Schuhmann *et al.*, 2008). PTS1- and PTS2-containing proteins are recognized in the cytosol by PEX5 and PEX7, respectively (reviewed in Hu *et al.*, 2012). In plants, the PEX7-PTS2-cargo complex requires PEX5 for peroxisome delivery (Hayashi *et al.*, 2005; Woodward and Bartel, 2005; Ramón and Bartel, 2010). Cargo-laden receptors dock with the PEX13 and PEX14 membrane proteins (reviewed in Azevedo and Schliebs, 2006), and yeast PEX5 enters the membrane to form a ligand-gated pore to deliver cargo into the peroxisomal matrix (Meinecke *et al.*, 2010). After delivery, yeast PEX5 in the membrane is ubiquitinylated (Kerssen *et al.*, 2006) to be either retrotranslocated and recycled for additional import (Collins *et al.*, 2000; Platta *et al.*, 2005), targeted for proteasomal degradation (Platta *et al.*, 2009), or degraded along with the peroxisome via peroxisome-specific autophagy (pexophagy) (Nordgren *et al.*, 2015; Zhang *et al.*, 2015; Sargent *et al.*, 2016).

Several ubiquitin-conjugating (UBC) enzymes and three RING-domain ubiquitin-protein ligases, PEX2, PEX10, and PEX12, are implicated in PEX5 ubiquitination. In yeast, the
PEX4 UBC enzyme works with PEX12 to mono-ubiquitinate PEX5 for recycling (Platta et al., 2007; Platta et al., 2009), and UBC4 cooperates with PEX2 to poly-ubiquitinate PEX5 for proteasomal degradation (Kiel et al., 2005; El Magraoui et al., 2012). PEX10 aids both ubiquitination processes (El Magraoui et al., 2012). In plants, it is assumed that PEX5 is similarly recycled or degraded; RING peroxins have ubiquitin-protein ligase activity in vitro (Kaur et al., 2013), and PEX5 accumulates in mutants defective in PEX4 or RING peroxins (Kao et al., 2016).

After PEX5 ubiquitination, the peroxisome-associated ATPases, PEX1 and PEX6, retrotranslocate PEX5 from the membrane back to the cytosol (reviewed in Grimm et al., 2016). PEX1 and PEX6 are members of a family of ATPases Associated with various cellular Activities (AAA proteins) with two AAA domains: AAA1 and AAA2. Unlike most AAA proteins, which are homohexamers, yeast PEX1 and PEX6 form a heterohexamer (Saffian et al., 2012) of alternating subunits arranged as a trimer of dimers (Blok et al., 2015; Ciniawsky et al., 2015; Gardner et al., 2015). Although AAA domains generally contain Walker A and B motifs involved in ATP binding and hydrolysis, respectively (reviewed in Grimm et al., 2016), PEX1 and PEX6 lack canonical Walker B motifs in AAA1, rendering AAA1 unable to hydrolyze ATP (reviewed in Grimm et al., 2016). However, ATP binding by the Walker A motif in yeast PEX1 AAA1 is required for PEX1-PEX6 interaction in vitro (Gardner et al., 2015). The AAA2 arginine fingers contact the neighboring subunit and contribute to PEX1-PEX6 complex formation (Ciniawsky et al., 2015) and hydrolysis of the neighboring ATP (Gardner et al., 2015). ATP hydrolysis by the PEX6 AAA2 domains drives conformational changes thought to promote PEX5 threading through the central pore (Ciniawsky et al., 2015). Although PEX5 is the only validated PEX1-PEX6 client, it has been suggested that matrix proteins including Arabidopsis isocitrate lyase might also be peroxisomal ATPase complex clients (Lingard et al., 2009; Burkhart et al., 2013).

The PEX6 N-terminal region tethers the PEX1-PEX6 hexamer to peroxisomes by binding to the N-terminal cytosolic domain of a tail-anchored peroxisomal membrane protein known as PEX26 in mammals (Tamura et al., 2006), PEX15 in yeast (Elgersma et al., 1997; Birschmann et al., 2003), and originally named APEM9 (Goto et al., 2011) or DAYU (Li et al., 2014) in Arabidopsis. In mammals, ATP hydrolysis by the PEX6 AAA2 Walker B domain is required for the PEX1-PEX6 hexamer to dissociate from PEX26 (reviewed in Fujiki et al., 2012). PEX15/26 is more than a simple tether; yeast PEX15 binding to the PEX1-PEX6 hexamer decreases ATPase activity (Gardner et al., 2015), perhaps preventing futile ATP hydrolysis prior to client recognition for extraction. The cytosolic domain of mammalian PEX26 also binds PEX14 (Tamura et al., 2014), hinting that PEX14-PEX26 interactions may help guide PEX5 from the docking complex to the export machinery.

Autosomal recessive mutations in PEX genes confer fatal peroxisomal biogenesis disorders (PBDs) in humans at a frequency of 1:50,000 in North America (reviewed in Braverman et al., 2016), and mutations in PEX1, PEX6, and PEX26 account for 65% of PBD patients (Waterham and Ebberink, 2012). PEX6 function is conserved from humans to plants; the Arabidopsis pex6-1 mutation (Zolman and Bartel, 2004) is equivalent to a causal mutation in a PBD patient (Zhang et al., 1999), and expressing a human PEX6 cDNA can restore Arabidopsis pex6-1 defects (Zolman and Bartel, 2004).
PEX1, PEX6, and PEX26 play key roles in plant peroxisome function. Reducing *Arabidopsis* PEX1, PEX6, or PEX26 mRNA via RNAi results in classical peroxisome-defective phenotypes including β-oxidation defects and inefficient import of peroxisome-targeted GFP (Nito *et al.*, 2007; Goto *et al.*, 2011). Two pex1, two pex6, and one pex26 missense alleles have been reported. The pex1-2 missense allele confers slight β-oxidation defects, whereas the pex1-3 missense allele is lethal when homozygous and confers peroxisomal defects when heterozygous (Rinaldi *et al.*, 2017). Like PEX1, PEX26 is essential for embryogenesis (Goto *et al.*, 2011). A pex26 missense allele, aberrant peroxisome morphology9-1 (apem9-1), mislocalizes matrix proteins in some tissues but lacks physiological defects (Goto *et al.*, 2011). A substitution in the pex6-1 AAA2 (Figure 1d, e) results in a stunted, pale-green plant with β-oxidation defects and low PEX5 levels (Zolman and Bartel, 2004). In contrast, the pex6-2 substitution N-terminal of AAA1 (Figure 1d) confers nearly wild-type phenotypes (Burkhart *et al.*, 2013). The distinct defects of peroxisomal ATPase complex mutants prompt the question of whether the phenotypic variations stem from differences in kind or degree.

Here, we describe three new mutant alleles (pex6-3, pex6-4, and pex26-1) of genes encoding the peroxin ATPase complex. These mutations conferred a range of peroxisomal defects, including low PEX5 levels accompanied by heterogeneous physiological defects and varied import and processing of peroxisome-targeted proteins. Overexpressing PEX5 alleviated some defects in pex6-3 but not in pex6-4 or pex26-1, suggesting ATPase complex functions beyond PEX5 recycling. Intriguingly, oil bodies persisted longer in pex6 and pex26 mutants than wild type during seedling development. These mutants illuminate roles for PEX6 and PEX26 in the import and export of peroxisomal proteins and reveal possible functions in oil body utilization.

**RESULTS**

*Arabidopsis* mutants with defective PEX6 or PEX26

Because peroxisomal enzymes β-oxidize IBA to IAA (reviewed in Strader and Bartel, 2011), mutations that reduce IBA-to-IAA conversion allow growth on otherwise inhibitory levels of IBA (reviewed in Bartel *et al.*, 2014). To isolate peroxisome-defective mutants, we screened the progeny of ethyl methanesulfonate-mutagenized *Arabidopsis thaliana* seeds for mutants resistant to the inhibitory effects of IBA on hypocotyl elongation in the dark and recovered several “hypocotyl resistant” (HR) mutants (Strader *et al.*, 2011). To enrich for mutants defective in matrix protein import rather than metabolic enzymes, we performed immunoblotting on leaf tissue to identify mutants with incomplete PTS2 processing. Because the PTS2 region is removed after import into the organelle (Helm *et al.*, 2007; Schuhmann *et al.*, 2008), PTS2 processing defects often indicate defective peroxisomal import. Among mutants displaying both IBA resistance and incomplete PTS2 processing, we recovered pex1-2 (Rinaldi *et al.*, 2017), pex12-1 (Kao *et al.*, 2016), several pex14 mutants (Fleming, 2016), and three isolates with mutations in PEX6 or PEX26 (Figure 1, 2).

Whole-genome sequencing of HR282 M4 lines revealed a G-to-A transition in PEX6 (*At1g03000*), resulting in a Gly817-to-Asp substitution (Figure 1c–e) between the AAA2 arginine finger residues (Figure 1e). We named this allele pex6-3. After two backcrosses, we...
again subjected pex6-3 DNA to whole-genome sequencing and confirmed the pex6-3 lesion, along with several linked mutations on chromosome 1 (Figure S1a).

Recombination mapping of HR119 revealed linkage to PEX6 on chromosome 1 (Figure 1b). Sequencing PEX6 from HR119 DNA revealed a C-to-T transition that causes an Ala867-to-Val substitution (Figure 1c–e). This alanine is C-terminal of the AAA2 and is conserved in human PEX1 (Figure 1e), and an identical substitution is reported in a human PBD patient (http://www.dbpex.org/). We named this allele pex6-4. After two backcrosses, we subjected pex6-4 DNA to whole-genome sequencing and confirmed the pex6-4 lesion, along with several linked mutations on chromosome 1 (Figure S1b).

We mapped the causal lesion in HR127 to chromosome 3 near PEX26 (Figure 2b). Sequencing PEX26 from HR127 DNA revealed a G-to-A transition in the splice-acceptor site at the 3’ end of intron 4 (Figure 2c). This pex26-1 mutation is expected to disrupt PEX26 splicing, which would prevent translation of the predicted C-terminal transmembrane domain (Figure 2d–e).

To visualize possible consequences of the pex6-3 and pex6-4 substitutions, we examined the location of analogous residues (Gly637 and Ala685, respectively) in human p97, a related homohexameric ATPase (Figure 1e) with high-resolution structural information available (Figure 1f). By examining the structure of p97 bound to ATPγS (Hanzelmann and Schindelin, 2017), we inferred that the pex6-3 Gly-to-Asp substitution between the arginine finger residues was positioned to impact interactions with the neighboring PEX1 ATP-binding pocket (Figure 1f), whereas the pex6-4 Ala-to-Val substitution alters a residue near the predicted PEX6 ATP-binding pocket (Figure 1f). Thus, the new PEX6 lesions altered residues predicted to reside on different faces of PEX6 protomers (Figure 1f) and were positioned to impact PEX1-PEX6 assembly or ATPase activity.

**Physiological defects in pex6 and pex26 mutants**

We compared pex6-3, pex6-4, and pex26-1 phenotypes to the two previously characterized pex6 alleles (Zolman and Bartel, 2004; Burkhart et al., 2013). Like pex6-1, we found that pex6-3, pex6-4, and pex26-1 displayed resistance to the inhibitory effects of IBA on both hypocotyl elongation in dark-grown seedlings (Figure 1a, 3a) and on root elongation in light-grown seedlings (Figure 3b). Also like pex6-1, the new mutants fully resisted IBA-induced lateral root formation, whereas pex6-2 was only partially resistant (Figure S2).

In addition to IBA β-oxidation, peroxisomes host fatty acid β-oxidation. Consequently, growth of peroxisome-defective mutants often can be improved by providing a fixed carbon source (e.g., sucrose). Like pex6-1, the new mutants grew better when sucrose-supplemented (Figure 3a), and pex6-4 displayed more marked hypocotyl elongation defects without sucrose than pex6-3 or pex26-1 (Figure 3a).

To determine whether the identified mutations in PEX6 and PEX26 were causal, we crossed the new pex6 mutants to a wild-type plant transformed with a CaMV 35S–driven HA-tagged PEX6 construct, and we transformed pex26-1 with a 35S:HA-PEX26 construct. We found that HA-PEX6 or HA-PEX26 restored sucrose independence and IBA sensitivity to pex6-3.
and pex6-4 or pex26-1 seedlings, respectively (Figure 3a–b), confirming that we had identified the causal mutations.

We used immunoblotting to investigate whether the mutations altered accumulation of PEX6 or the PEX6-interacting ATPase PEX1. PEX6 and PEX1 levels resembled wild type in pex6-1, pex6-2, pex6-3, pex6-4, and pex26-1 (Figure 3c), suggesting that these mutations do not markedly influence PEX6 or PEX1 stability.

Impaired matrix protein processing and decreased PEX5 levels in pex6 and pex26 mutants

To test whether the physiological defects were accompanied by inefficient import of peroxisome-targeted proteins, we used immunoblotting to examine processing of two PTS2 proteins: thiolase and peroxisomal malate dehydrogenase (PMDH). Like pex6-1, we found that pex6-3, pex6-4, and pex26-1 all displayed incomplete PMDH processing in seedlings (Figure 3c–d) and mature leaves (Figure 3e), with pex6-3 seedlings generally showing more severe PMDH-processing defects than pex6-4 or pex26-1 (Figure 3c–d). Interestingly, the pex26-1 PMDH PTS2-processing defect was more apparent in mature leaves (Figure 3e) than in seedlings (Figure 3c–d), suggesting that pex26-1 import worsens with age. In contrast, seedling thiolase-processing defects were apparent in pex6-4 and pex26-1 but were not usually detected in pex6-3 (Figure 3c–d). As expected, PTS2 processing was largely or fully restored in pex6 and pex26 mutants expressing the corresponding wild-type gene (Figure 3c).

pex6-1 displays low PEX5 levels (Zolman and Bartel, 2004), presumably because PEX5 marooned in the peroxisomal membrane (Ratzel et al., 2011) is poly-ubiquitinated and degraded by the proteasome (Kao et al., 2016). In contrast, PEX5 levels are not reduced in pex6-2 (Burkhart et al., 2013). Given this disparity, we investigated PEX5 levels in our mutants, and like pex6-1, we found that pex6-3, pex6-4, and pex26-1 had low PEX5 levels in seedlings and in rosette leaves of mature plants (Figure 3c–f) that were fully or partially restored by expressing HA-PEX6 or HA-PEX26, respectively (Figure 3c). PEX5 levels generally appeared lower in pex6-3 than in pex6-4 or pex26-1 (Figure 3c–f). These decreased PEX5 levels suggest that PEX5 degradation is heightened in these new pex6 and pex26 mutants. As in pex6-1 (Kao and Bartel, 2015), PEX5 levels were increased by treating pex6-3, pex6-4, and pex26-1 with the proteasome inhibitor MG132 (Figure 3f), suggesting that the proteasome contributes to PEX5 degradation in these mutants.

Growth defects of pex6 and pex26 mutants

The pex6-3, pex6-4, and pex26-1 mutants displayed general growth defects, reflecting the importance of peroxisome function in multiple developmental stages. After 1 week on sucrose-supplemented medium, pex6-1, pex6-3, pex6-4, and pex26-1 seedlings had somewhat shorter roots than wild type (Figure 3b, 4a). In addition, 2-week-old pex6-3 and pex6-4 were slightly pale, like pex6-1 (Figure 4b). Also like pex6-1, pex6-3 plants were smaller than pex6-4 or pex26-1 plants at maturity (Figure 4c), consistent with the more dramatically reduced PEX5 levels in pex6-1 and pex6-3 leaves (Figure 3e).
A PEX26 cytosolic-domain variant confers matrix protein processing defects

The pex26-1 splice-site mutation occurs prior to the exon encoding the transmembrane domain and might allow a truncated PEX26 variant to accumulate. PEX26 tethers PEX1-PEX6 to peroxisomes (Goto et al., 2011), and the cytosolic domain of the yeast PEX26 homolog reduces in vitro PEX1-PEX6 ATPase activity (Gardner et al., 2015), suggesting that an N-terminal PEX26 cytosolic domain might sequester PEX1-PEX6 away from peroxisomes or impair PEX1-PEX6 catalysis. To test if the predicted pex26-1 N-terminal fragment was toxic because it lacked the C-terminal peroxisomal anchor, we compared wild-type lines producing full-length HA-PEX26 with two variants lacking the transmembrane domain (Figure S3a): HA-pex26ΔTM1 (aa 1–191), which mimics the predicted pex26-1 protein, and HA-pex26ΔTM2, which includes the entire region N-terminal of the transmembrane domain (aa 1–262). Although we could detect both HA-pex26ΔTM proteins in seedling extracts (Figure S3d–e), these lines did not generally display growth defects or IBA resistance (Figure S3b–c). However, HA-pex26ΔTM2 line B displayed slightly impaired elongation without sucrose and slightly less responsiveness to the proliferative effects of IBA on lateral root production (Figure S3b–c). Interestingly, expressing HA-pex26ΔTM2 conferred PMDH-processing defects (Figure S3d–e). The failure of 35S:HA-pex26ΔTM1 to confer similar dominant-negative effects (Figure S3) suggests that pex26-1 defects result from reduced PEX26 function rather than interference with peroxisome function by a truncated cytosolic domain.

Impairing PEX2, but not other peroxisomal ubiquitination components, partially alleviates pex26-1 defects

Several mutants defective in the peroxisomal ubiquitination machinery, including pex4-1 (Zolman et al., 2005), pex2-1 (Burkhart et al., 2014), pex10-2 (Burkhart et al., 2014), and pex12-1 (Kao et al., 2016) display elevated PEX5 levels (Kao et al., 2016), suggesting that the PEX4 ubiquitin-conjugating enzyme and the PEX2, PEX10, and PEX12 ubiquitin-protein ligases can promote PEX5 degradation. Indeed, both pex4-1 (Ratzel et al., 2011) and pex2-1 (Burkhart et al., 2014) restore PEX5 levels in pex6-1. We used double mutants to examine the epistatic relationships between pex26-1 and ubiquitination machinery mutants. These mutants restored PEX5 levels in pex26-1 (Figure 5c–e), indicating that the corresponding enzymes directly or indirectly promote the excessive PEX5 degradation observed in pex26-1. However, the physiological defects of pex26-1 seedlings also carrying pex4-1, pex10-2, or pex12-1 mutations were not rescued; these double mutants displayed retained (Figure 5a) or exacerbated (Figure 5b) IBA resistance, grew poorly on media lacking sucrose (Figure 5a), and processed PTS2 proteins less effectively (Figure 5c–d). For example, the clear PEX5 elevation in pex26-1 pex10-2 was accompanied by growth defects even on sucrose-supplemented medium (Figure 5a–b) and dramatically reduced PTS2 processing (Figure 5c–e), indicating that peroxisome dysfunction was heightened despite restored PEX5 levels.

Stability of the peroxin RING complex is dependent upon the presence of all three proteins in yeast (Rayapuram and Subramani, 2006; El Magraoui et al., 2012). Similarly in Arabidopsis, PEX10 levels are reduced in pex2-1, pex10-2, and pex12-1 (Kao et al., 2016). The increased PEX10 level in pex4-1 (Figure 5f, Kao et al., 2016) implies that PEX4-
dependent ubiquitination contributes to PEX10 retrotranslocation and subsequent
proteasomal degradation. Interestingly, the low pex10 levels in pex10-2 were partially
restored by pex26-1 (Figure 5f), suggesting that PEX26 promotes pex10 degradation in
pex10-2. Thus in addition to PEX5, PEX10 might be an export client of the peroxisomal
ATPase complex.

Like the other double mutants, pex26-1 pex2-1 remained IBA resistant (Figure 5a–b) and
displayed elevated PEX5 (Figure 5c–e) and exacerbated thiolase-processing defects in 8-d-
old light-grown seedlings (Figure 5d). Unlike the other double mutants, however, pex2-1
partially alleviated pex26-1 growth defects without sucrose (Figure 5a). This partial
suppression implies that reducing PEX2-mediated ubiquitination restores some peroxisome
functioning in young pex26-1 seedlings.

**PEX5 membrane association is elevated in pex6 mutants**

The reduced PEX5 levels in our new pex6 and pex26 alleles suggested that similar to pex6-1
(Ratzel et al., 2011), PEX5 might be excessively retained in the peroxisome membrane. We
tested this idea by fractionating seedling extracts using centrifugation and monitoring
peroxin localization to the cytosol (containing HSC70) or organelles (containing
mitochondrial ATP synthase). As previously observed (Kao and Bartel, 2015), the
membrane peroxin PEX14 was predominantly in the pellet, the cytosolic receptor PEX7 was
largely soluble, and PEX5 was found in both fractions in wild type (Figure 6). Protein
distribution in pex6-3 and pex6-4 resembled pex6-1, with most of the recovered PEX5 in the
pellet (Figure 6), consistent with a defect in removing PEX5 from the peroxisomal
membrane. However, PEX5 distribution in pex26-1 resembled wild type (Figure 6),
suggesting that pex26-1 defects in PEX5 retrotranslocation, if present, are not sufficient to
detect using this assay.

**Overexpressing PEX5 affects pex6 and pex26 mutants differently**

PEX5 levels are low in pex6-1, and overexpressing PEX5 partially rescues pex6-1 sucrose
dependence, PTS2 processing, and growth defects (Zolman and Bartel, 2004; Ratzel et al.,
2011). Similarly, we found that overexpressing PEX5 in pex6-3 partially alleviated
physiological defects of dark- and light-grown seedlings (Figure 7a–b). In contrast, pex6-4
defects were unaltered, and the sucrose dependence and IBA resistance of pex26-1 were
heightened by PEX5 overexpression (Figure 7a–b). These mutants also showed dissimilar
PTS2-processing responses to PEX5 overexpression. In contrast to the partially restored
PMDH processing in pex6-1 35S:PEX5 (Figure 7d), overexpressing PEX5 decreased
thiolase processing in pex6-4 and worsened both PMDH and thiolase processing in pex26-1
(Figure 7c–d). The contrasting physiological and molecular consequences of PEX5
overexpression in various pex6 and pex26 alleles suggest that low PEX5 levels is not the
sole shortcoming in these mutants. We attempted to determine if excess PEX5 was
differently distributed in the various mutants, but did not detect PEX5 localization
differences following centrifugal fractionation (Figure S4).
Reduced import of peroxisome-targeted GFP in pex6 and pex26 mutants

The PTS2-processing defects in pex6 and pex26 (Figure 3c–e, 5c–e, 7c–d) suggest defects in peroxisome-targeted matrix protein import. To directly examine import, we monitored localization of a peroxisome-targeted GFP derivative (GFP-PTS1). Like pex6-1, both pex6-3 and pex6-4 inefficiently imported GFP-PTS1 into seedling peroxisomes, displaying partially cytosolic GFP fluorescence in cotyledon and hypocotyl cells (Figure 8). Consistent with the minor PTS2-processing defects (Figure 3c–e, 5c–e, 7c–d), pex26-1 seedlings displayed predominantly GFP puncta with only slight cytosolic mislocalization (Figure 8). Interestingly, some peroxisomes were clustered in 5-d-old pex6-1, pex6-3, pex6-4, and pex26-1 seedlings, unlike the more dispersed peroxisomes in wild type (Figure 8c).

Oil body retention in pex6 and pex26 mutants

During lipid mobilization, peroxisomes cluster with oil bodies (Hayashi et al., 2001; Thazar-Poulot et al., 2015), which are abundant in germinating wild-type seedlings and become scarce after about 3 days (Siloto et al., 2006; Rinaldi et al., 2016). sdpl (Cui et al., 2016) and other fatty acid β-oxidation mutants (Rinaldi et al., 2016) metabolize fatty acids slowly and retain oil body-peroxisome clusters in older seedlings. The clustered peroxisomes in pex6 and pex26 seedlings (Figure 8c) prompted us to examine oil bodies. To monitor lipid utilization in our mutants, we stained neutral lipids with Nile red (Greenspan et al., 1985). Unlike wild-type and pex6-2 seedlings, which displayed only sparse oil bodies by 5 days, we found that oil bodies remained numerous in 5-d-old pex6-1, pex6-3, pex6-4, and pex26-1 mutants and that peroxisome clusters often included oil bodies (Figure 8).

We tested whether increasing PEX5 levels would reduce oil body persistence in these mutants. As expected, wild-type cotyledon cells had few if any oil bodies by day 5, regardless of PEX5 overexpression (Figure 7e). Overexpressing PEX5 largely ameliorated the oil body persistence in pex6-1 and pex6-3 (Figure 7e). In contrast, the oil body persistence in pex6-4 and pex26-1 was not corrected by PEX5 overexpression (Figure 7e), again suggesting distinct underlying molecular defects in these alleles.

DISCUSSION

Arabidopsis peroxin mutants defective in the ATPase complex

PEX1, PEX6, and PEX26 extract PEX5 from the peroxisomal membrane to allow additional rounds of import (Figure 9a). Several viable Arabidopsis pex1 (Rinaldi et al., 2017), pex6 (Zolman and Bartel, 2004; Burkhart et al., 2013), and pex26 (Goto et al., 2011) mutants have been characterized, and comparing these alleles with the pex6-3, pex6-4, and pex26-1 alleles reported here reveals disparate phenotypes that expand our understanding of PEX6 and PEX26 functions in plants.

Arabidopsis PEX26 is essential for viability; PEX26 null mutations confer embryonic lethality (Goto et al., 2011; Li et al., 2014). In contrast, apem9-1 (Goto et al., 2011) and pex26-1 (Figure 2) are viable, indicating that these are hypomorphs. pex26-1 was IBA resistant and partially sucrose dependent (Figure 2a, 3a–b, 5a–b, 7a–b), whereas apem9-1 seedlings respond like wild type to a synthetic IBA analog and grow normally without
sucrose (Goto et al., 2011), suggesting that the pex26-1 splicing mutation more severely reduces PEX26 function than the apeM9-1 missense mutation.

Unlike PEX1 and PEX26, PEX6 null mutations have not been reported. All four Arabidopsis pex6 mutations are viable missense alleles (Figure 1d–e) that still produce pex6 protein (Figure 3c), consistent with the possibility that these mutants retain partial PEX6 function. pex6-2 carries a mutation altering a residue N-terminal of AAA1 and resembles wild type in most assays (Figure 1a, 3–4, 8; Burkhart et al., 2013). pex6-1, pex6-3, and pex6-4 confer more severe defects and alter conserved residues in or near the AAA2 domain (Figure 1d–f). Similarly, the two Arabidopsis pex1 missense alleles alter conserved residues in the PEX1 AAA2 domain (Rinaldi et al., 2017), highlighting the importance of this domain.

The 52% identity between the AAA2 region of Arabidopsis PEX6 and human p97 (Figure 1e), for which high-resolution structural information is available (Hanzelmann and Schindelin, 2017), allows speculation on the structural bases for pex6 mutant defects. pex6-1 alters a conserved residue in the second pore loop (Figure 1e) near the PEX1-PEX6 interaction surface (Figure 1f), suggesting that pex6-1 might impair client translocation or hexamer assembly. pex6-3 alters a conserved Gly residue between the arginine fingers (Figure 1e–f), perhaps disturbing nucleotide binding or hydrolysis by the neighboring PEX1 subunit. In contrast, pex6-4 alters a conserved residue near the predicted PEX6 ATP-binding pocket (Figure 1f), which might impair ATPase activity of PEX6 subunits without impacting PEX6-PEX1 interactions. Both pex6-1 and pex6-4 are analogous to mutations in human PBD patients (http://www.dbpex.org/; Zhang et al., 1999), underlining the conservation of PEX6 structure and function.

Imperfect correlations between β-oxidation and PTS2-processing defects

Because seedling growth requires matrix enzyme import, and because PTS2 processing occurs after import, we expect the extent of GFP-PTS1 import and PTS2-processing defects to mirror the severity of physiological defects. Like pex6-1, pex6-3 and pex6-4 displayed both diffuse cytosolic GFP-PTS1 and puncta, whereas pex26-1 displayed more complete GFP-PTS1 import (Figure 8). We also monitored peroxisomal import via PTS2 processing of thiolase and PMDH. Thiolase acts directly in the last step of β-oxidation, whereas PMDH oxidizes NADH produced during β-oxidation and other peroxisomal metabolic reactions (Pracharoenwattana et al., 2007). PMDH-processing defects generally appear more severe than thiolase-processing defects in pex mutants (Lingard and Bartel, 2009; Ramón and Bartel, 2010; Monroe-Augustus et al., 2011; Burkhart et al., 2014; Woodward et al., 2014; Kao et al., 2016). Similarly, pex6-1 and pex6-3 displayed more severe defects in processing PMDH than thiolase (Figure 3c–e, 7d). However, PMDH processing was not perfectly correlated with thiolase processing in the mutants examined here. Unlike pex6-1 and pex6-3, 4-d-old pex6-4 and pex26-1 seedlings displayed a greater defect in processing thiolase than PMDH (Figure 3d, 5e).

Interestingly, sucrose dependence tracked more closely with defects in processing of thiolase than PMDH. pex6-1, pex6-4, and to a lesser extent pex26-1 were sucrose dependent (Figure 3a, 7a) and showed substantial (pex6-1 and pex6-4) or minor (pex26-1) thiolase-processing
defects as seedlings (Figure 3c–d, 7d). In contrast, pex6-2 and pex6-3 grew relatively well without sucrose (Figure 3a, 7a) and processed thiolase completely or nearly completely (Figure 3c–d, 7c–d). This phenotypic disparity hints that the various mutations preferentially impact different PEX1-PEX6-PEX26 functions rather than disabling a single function to varying degrees.

**Peroxisome – oil body relationships**

Because the pex6 and pex26 mutants did not completely prevent GFP-PTS1 import into peroxisomes (Figure 8), we could use this reporter to follow peroxisome positioning in the mutants. We observed clusters of peroxisomes associated with persisting oil bodies in pex6-1, pex6-3, pex6-4, and pex26-1 seedlings (Figure 8). Whereas oil bodies are rapidly consumed following germination in wild type (Siloto et al., 2006), we still detected oil bodies in pex6-1, pex6-3, pex6-4, and pex26-1 cotyledons 5 days after plating (Figure 7e, 8). This oil body persistence may contribute to or reflect the sucrose dependence observed in these alleles. Indeed, when PEX5 overexpression alleviated sucrose dependence, persistent oil bodies were no longer detected; pex6-1 35S:PEX5 and pex6-3 35S:PEX5 lacked persistent oil bodies and resembled wild type when grown without sucrose, whereas excessive PEX5 did not confer sucrose independence or oil body utilization in pex6-4 or pex26-1 (Figure 7a, e). In spite of restored sucrose independence and oil body utilization, PTS2 processing in pex6-1 and pex6-3 was not fully restored (Figure 7d), indicating that some malfunctions remain.

There are several reasons that oil body utilization might be slowed in these mutants. Inefficient peroxisomal import of β-oxidation enzymes slows fatty acid β-oxidation, and perhaps this slowing negatively impacts oil body consumption. Alternatively, perhaps PEX6 and PEX26 directly contribute to oil body utilization. pex6-1, pex6-3, pex6-4, and pex26-1 displayed inefficient matrix protein import (Figure 8), consistent with the former possibility. However, the inability of elevated PEX5 levels to restore oil body usage in pex6-4 and pex26-1 (Figure 7e) hints that the peroxisomal ATPase complex may function more directly in oil body consumption.

Although peroxisomes and oil bodies interact in *Arabidopsis* (Hayashi et al., 2001; Schumann et al., 2003; Thazar-Poulot et al., 2015), the molecular details of the relationship are incompletely understood. Peroxisome-oil body clustering is heightened when *Arabidopsis* seedlings are grown without sucrose (Cui et al., 2016). We observed peroxisomes nestled around persisting oil bodies in pex6-1, pex6-3, pex6-4, and pex26-1 even when sucrose-supplemented (Figure 8). SDP1 localizes to both peroxisomes and oil bodies (Eastmond, 2006; Thazar-Poulot et al., 2015), and it has been suggested that PXA1 anchors peroxisome-oil body interaction (Cui et al., 2016). Screens for disturbed oil body morphology and peroxisomal clusters near oil bodies yielded sdp1 (Cui et al., 2016) and a variety of β-oxidation mutants (Cui et al., 2016; Rinaldi et al., 2016). Although our screen was not designed to recover mutants displaying oil body persistence, pex6-3, pex6-4, and pex26-1 phenotypes further illuminate the relationship of peroxisomes and oil bodies in *Arabidopsis*. It will be interesting to visualize oil bodies in other peroxin mutants to
determine if oil body persistence is symptomatic of general peroxisome dysfunction or specific to mutants in the peroxisomal ATPase complex.

**Implications for PEX5 retrotranslocation**

Analysis of peroxisome-defective mutants from a variety of organisms indicates that peroxisomal matrix protein import requires PEX5 recycling by the peroxisomal ATPase complex (reviewed in Grimm et al., 2016; Figure 9a). Like human pex6 patient-derived cell lines (Dodt and Gould, 1996), Arabidopsis pex6-1 has low PEX5 levels (Zolman and Bartel, 2004), probably because PEX5 accumulating in the membrane (Ratzel et al., 2011) in the absence of efficient retrotranslocation is degraded by the proteasome (Kao and Bartel, 2015) rather than recycled. We found that pex6-3 and pex6-4 also had reduced PEX5 levels (Figure 3c–f) that were slightly increased by proteasome inhibition (Figure 3f), and that PEX5 resided predominantly in the membrane in these mutants (Figure 6). These results suggest that pex6-3 and pex6-4 also inefficiently retrotranslocate PEX5, leading to PEX5 poly-ubiquitination and proteasomal degradation.

We tested whether low PEX5 levels contributed to the peroxisome-related defects of the new pex6 and pex26 mutants by overexpressing PEX5. As with pex6-1 (Zolman and Bartel, 2004), overexpressing PEX5 partially ameliorated pex6-3 physiological defects (Figure 7a) and oil body utilization (Figure 7e), suggesting that heightened PEX5 degradation contributes to pex6-3 deficiencies. However, pex6-4 defects were not mitigated and pex26-1 defects were worsened when PEX5 was overexpressed (Figure 7), indicating that pex6-4 and pex26-1 defects do not exclusively stem from low PEX5 levels and hinting at differential impairment of PEX6 and PEX26 function(s) in these mutants.

To genetically test whether ubiquitination played a role in PEX5 diminution in pex26-1, we introduced mutations in the peroxisome-associated ubiquitination machinery. We found that pex4-1, pex10-2, and pex12-1 each increased PEX5 levels in pex26-1 (Figure 5c–e). Like overexpressing PEX5 (Figure 7a–d), these mutations worsened the physiological and molecular defects of pex26-1 (Figure 5). Similarly, pex4-1 exacerbates the physiological and molecular defects of pex6-1 despite restoring PEX5 levels (Ratzel et al., 2011). These data add to accumulating evidence (Ratzel et al., 2011; Burkhart et al., 2014; Kao and Bartel, 2015; Kao et al., 2016) that PEX5 lingering in the peroxisomal membrane after cargo delivery is detrimental to peroxisome function (Figure 9b).

One mechanism by which PEX5 retained in the membrane might impair peroxisome function is by triggering autophagy of peroxisomes, or pexophagy (reviewed in Young and Bartel, 2016). Peroxisomes are degraded via autophagy in yeast and mammalian cells with PEX1, PEX6, or PEX26 defects (Nuttall et al., 2014; Law et al., 2017), and mammalian PEX2 promotes PEX5 poly-ubiquitination and pexophagy under starvation conditions (Sargent et al., 2016). Along with increasing PEX5 levels (Figure 5c–e), the Arabidopsis pex2-1 mutation improved pex26-1 seedling growth in the absence of sucrose (Figure 5a). Similarly, pex2-1 restores PEX5 levels and slightly improves pex6-1 growth (Burkhart et al., 2014). Assuming that PEX2 promotes poly- rather than mono-ubiquitination of PEX5 in Arabidopsis (Figure 9a) as it does in yeast (Platta et al., 2009) and mammals (Sargent et al., 2016), this suppression suggests that PEX5 poly-ubiquitination contributes to peroxisome...
dysfunction in pex26-1, perhaps by triggering pexophagy (Figure 9b). The exacerbated defects conferred by PEX5 overexpression in pex6-2 (Burkhart et al., 2013), pex6-4 (Figure 7), pex26-1 (Figure 7), and pex1 mutants (Rinaldi et al., 2017) are consistent with the possibility that this pexophagy is heightened in these mutants when PEX5 is overexpressed. It will be interesting to learn whether preventing autophagy ameliorates peroxisome-related defects in Arabidopsis pex6 and pex26 mutants, as has recently been observed in a pex1 mutant (Rinaldi et al., 2017).

Why then is PEX5 overexpression beneficial rather than detrimental in pex6-1 and pex6-3 mutants? We speculate that in addition to extracting mono-ubiquitinated PEX5, PEX1-PEX6 might also remove poly-ubiquitinated clients, including PEX5, from the peroxisome for proteasomal degradation (Figure 9a). This hypothesis stems from the similarity of PEX1-PEX6 to p97 (Figure 1e), which acts as a “segregase” to extract clients from the ER membrane without removing the poly-ubiquitin that will promote subsequent proteasomal degradation of the client (reviewed in Xia et al., 2016). Unlike p97, the central pore of PEX1-PEX6 appears large enough to thread an unfolded (and presumably de-ubiquitinated) client protein (reviewed in Saffert et al., 2017), which would allow PEX5 recycling. In addition to this recycling function, we speculate that PEX1-PEX6 may also function remove poly-ubiquitinated PEX5 from the membrane without de-ubiquitination, similarly to p97 action (Figure 9a). If the pex6-1 and pex6-3 alleles retain the function of removing poly- but not mono-ubiquitinated PEX5, this hypothesis would explain both the lower PEX5 levels in these alleles (Figure 3c–f, 7c–d) and the unusual amelioration of pex6-1 and pex6-3 defects by PEX5 overexpression (Figure 7).

Interestingly, the pex6-2 allele emerged from a screen for stabilized peroxisomal matrix proteins but lacks most hallmarks of peroxisome dysfunction and is able to complement the pex6-1 mutant (Burkhart et al., 2013). In contrast to the decreased PEX5 levels of the other pex6 alleles, PEX5 levels appear to be slightly elevated in pex6-2 (Figure 3c–f). It is tempting to speculate that pex6-2 maintains the ability to extract mono-ubiquitinated PEX5 but is defective in extracting poly-ubiquitinated clients.

In conclusion, the disparate phenotypes of pex6 and pex26 mutants described here, including opposite responses to excessive levels of the PEX5 client protein, suggest multiple functions for the peroxisomal ATPase complex. These functions may include not only canonical PEX5 recycling, but also facilitating PEX5 degradation, removing additional client proteins, promoting oil body mobilization, and preventing pexophagy. The ability to detect ubiquitinated PEX5 forms in Arabidopsis, which has not yet been reported, would aid in testing and further developing these hypotheses. In addition, development of peroxisomal membrane markers would be useful to monitor peroxisome numbers and localization in various mutants regardless of matrix protein import capacity. It will be interesting to learn whether PEX6 or PEX1 missense alleles in other eukaryotes, such as the causal mutations in human peroxisome biogenesis disorders, are similarly affected in a subset of ATPase functions.
EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The Arabidopsis thaliana accession Columbia-0 (Col-0) was used as wild type, and mutants were in the Col-0 background. pex2-1 (Burkhart et al., 2014), pex4-1 (Zolman et al., 2005), pex6-1 (Zolman and Bartel, 2004), pex6-2 (Burkhart et al., 2013), pex10-2 (Burkhart et al., 2014), and pex12-1 (Kao et al., 2016) were previously described. pex6-3, pex6-4, and pex26-1 single mutants were backcrossed once or twice prior to phenotypic assays. Seedlings were transferred from plates to soil (SunGro MetroMix 366) after 1–2 weeks and grown under constant white light at 22°C.

pex6-1 35S:PEX5 and wild type 35S:PEX5 express a PEX5 cDNA and were previously described (Zolman and Bartel, 2004). pex6-3, pex6-4, and pex26-1 were crossed to wild type 35S:PEX5. Plants with transgene were selected using Basta resistance and PCR-amplifying PEX5 using intron-spanning primers (Table S1).

pex6-1 35S:GFP-PTS1 was previously described (Zolman and Bartel, 2004). To obtain lines expressing peroxisome-targeted GFP, pex6-3, pex6-4, and pex26-1 were crossed to wild type carrying 35S:GFP-PTS1 (Zolman and Bartel, 2004). PCR-amplification using primers annealing to the promoter and GFP (Table S1) was used to track the transgene.

For physiological assays, seeds were surface sterilized in 50% (v/v) commercial bleach supplemented with 0.01% (v/v) Triton X-100, stratified 1–2 d at 4°C, and plated on plant nutrient (PN) medium (Haughn and Somerville, 1986) solidified with 0.6% (w/v) agar. Media were supplemented with 0.5% (w/v) sucrose (PNS) and IBA from an ethanol-dissolved stock solution as indicated. Seedlings were grown on plates wrapped in gas-permeable surgical tape at 22°C. For root elongation experiments, plates were incubated for 8 d under continuous light filtered through yellow long-pass filters to slow indolic compound breakdown (Stasinopoulos and Hangarter, 1990). For hypocotyl elongation experiments, plates were incubated for 1 day in yellow-filtered light followed by 4 d in darkness. For lateral-root assays, 4-d-old seedlings grown under yellow-filtered light on PNS were transferred to PNS with or without 10 µM IBA; lateral roots emerged from the epidermis were counted 4 d later. Physiological experiments were repeated at least twice with similar results.

Mutant isolation and recombination mapping

The pex6-3, pex6-4, and pex26-1 mutants were isolated by screening progeny of ethyl methanesulfonate-mutagenized Col-0 on PNS with 30 µM IBA for individuals with elongated dark-grown hypocotyls (Strader et al., 2011). Putative mutants were transferred to PNS in the light for recovery prior to transfer to soil. Immunoblotting on leaf tissue of adult plants was used to monitor PTS2 processing.

For recombination mapping of HR119 (pex6-4) and HR127 (pex26-1), F2 individuals from outcrosses to Landsberg erecta were genotyped using PCR-based polymorphic markers (Table S2). Candidate genes were PCR-amplified from mutant DNA and sequenced (Lone Star Labs, Houston, TX) to find the pex6-4 and pex26-1 lesions. Mutations were genotyped.
by PCR-amplification and restriction digestion using primers and enzymes listed in Table S1.

Whole-genome sequencing

Seedlings were grown under continuous light for 11 or 16 d on filter paper on PNS. Genomic DNA was prepared as described (Thole et al., 2014). For sequencing backcrossed lines, seedlings from several lines were pooled to reduce the appearance of homozygous non-causal background mutations. Sequencing was performed at the Genome Technology Access Center at Washington University in St. Louis using an Illumina HiSeq2000 sequencer and was analyzed as described (Farmer et al., 2013) to find EMS-consistent lesions in predicted coding sequences, introns, and untranslated regions that were absent in our lab version of Col-0. For both pex6 mutants, at least 87% of the genome had 10-fold coverage.

DNA constructs and plant transformation

A PEX6 cDNA in pENTR223 (stock G21748) and a PEX26 cDNA in pENTR/SD-DTOPO (stock U16579) were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University and transferred into the pEG201 destination vector (Earley et al., 2006) using clonase (Invitrogen) to give 35S:HA-PEX6 and 35S:HA-PEX26.

For the truncated PEX26 constructs, two PEX26 cDNA regions lacking the encoded transmembrane domain were separately amplified using PEX26-11 (5'-CACCATGGAGGCAACTGATATTTGGGGAGA-3') with PEX26-12 (5'-TCATCGCCTTCTCAGCAATGCAGCTTT-3') or PEX26-11 with PEX26-13 (5'-TCAGGTATTACCAACCTTGAGGCTTAGG-3') to create cDNAs lacking the transmembrane region, pex26ΔTM1 and pex26ΔTM2, respectively. These cDNAs were subcloned into the pENTR-gus entry vector (Invitrogen) prior to transfer into the pEG201 destination vector (Earley et al., 2006) using clonase (Invitrogen) to create 35S:HA-pex26ΔTM1 and 35S:HA-pex26ΔTM2.

Constructs were transformed into Agrobacterium tumefaciens GV3101 (pMP90) (Koncz and Schell, 1986), which was used to transform Arabidopsis plants using the floral dip method (Clough and Bent, 1998). 35S:HA-PEX26 was transformed into pex26-1 and wild type, 35S:HA-PEX6 was transformed into wild type before crossing into pex6-3 and pex6-4, and 35S:HA-pex26ΔTM1 and 35S:HA-pex26ΔTM2 were transformed into wild type. Transformants were selected on PNS plates containing 8–10 µg/mL Basta, and homozygous lines were identified by following Basta resistance in the progeny.

Immunoblot analysis

Tissue was processed for immunoblotting as described (Kao and Bartel, 2015), except that protein transfer was for 50 min and membranes were air dried before blocking in 8% non-fat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20). Primary antibodies were as follows: rabbit anti-PEX1 (1:200, Rinaldi et al., 2017), anti-PEX5 (1:100, Zolman and Bartel, 2004), anti-PEX6 (1:800–1000, Ratzel et al., 2011), anti-PEX7 (1:2000, Ramón and Bartel, 2010), anti-PEX10 (1:500, Burkhart et al., 2014), anti-PEX14 (1:10,000, Agrisera.
AS08), anti-thiolase (1:2,500-1:5000, Lingard et al., 2009), anti-PMDH2 (1:2,000, Pracharoenwattana et al., 2007); mouse anti-mitochondrial ATP synthase subunit α (1:2,000, MitoScience MS507) and anti-HSC70 (1:50,000–1:100,000, SPA-817, StressGen Biotechnologies); and rat anti-HA (1:100–1:500, Roche clone 3F10). Horseradish peroxidase-conjugated secondary antibodies (1:5,000) were incubated for 3–6 hours before washing and imaging using WesternBright ECL substrate (Advansta). Films were scanned with a flatbed scanner, and bands were quantified using NIH image J. Membranes were sequentially probed with various antibodies without stripping. Immunoblotting experiments were repeated at least twice with similar results.

Confocal fluorescence microscopy

Seedlings were grown under continuous light on PNS. Germination was scored 2 days after plating, and cotyledons from individuals with emerged radicles at day 2 were stained with 5 µg/mL Nile red and imaged on day 5. Fluorescence was captured using a Carl Zeiss 710 confocal microscope with a 63X oil-immersion objective and a Meta detector. Each image is an average of 4 images with 8-bit depth. Confocal microscopy experiments were repeated at least four times with similar results.

Cell fractionation

An approximately equal mass (±0.01 g) of seedlings was collected for each genotype after growth for 1 d in light and 5 or 6 d in darkness. Tissue was processed as described (Kao and Bartel, 2015). Fractionation experiments were repeated at least twice with similar results.

Statistical analysis

One-way ANOVA with Duncan’s test was used to assess statistical significance (SPSS Statistics software). Significant differences (P < 0.001) are noted by different letters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Peroxisomes and oil bodies are critical for seedling development and have extensive physical and metabolic interactions; however, peroxins have not been implicated in this relationship. We found that pex6 and pex26 mutants with defects in the peroxisomal ATPase complex display a range of allele-specific peroxisome dysfunctions including defects in oil body utilization during Arabidopsis seedling development.
Figure 1. Identification of \textit{pex6-3} and \textit{pex6-4}

(a) \textit{pex6-3} and \textit{pex6-4} are IBA resistant. Bars indicate mean hypocotyl lengths of dark-grown seedlings (n ≥ 12). Error bars indicate SD; different letters above bars indicate significant differences (one-way ANOVA, P < 0.001).

(b) Recombination mapping localized the HR119 (\textit{pex6-4}) mutation on chromosome 1. Numbers indicate recombinant chromosomes over chromosomes assessed at the indicated markers.
(c) PEX6 gene diagram showing exons (rectangles), introns (lines), and the locations of pex6 mutations. The pex6-3 G-to-A transition yields a Gly-to-Asp substitution. The pex6-4 C-to-T transition yields an Ala-to-Val substitution.

(d) PEX6 protein schematic showing the locations of the two conserved AAA domains (gray); the Walker A (WA), Walker B (WB), and arginine fingers (asterisks); and Arabidopsis pex6 mutations (aqua), including previously described missense alleles, pex6-1 (Zolman and Bartel, 2004) and pex6-2 (Burkhart et al., 2013).

(e) PEX6 is a conserved AAA ATPase. The alignment shows the AAA2 regions of Arabidopsis thaliana (At) and human (Homo sapiens; Hs) PEX6 and PEX1 with human p97 highlighting the Walker A and B domains, arginine fingers (asterisks), and pex6 missense alleles. p97 pore loops 1 and 2 are underlined in gray, and pore loop 2 residues that are not ordered in the structure used to generate panel f (Hanzelmann and Schindelin, 2017) are in red. The alignment was generated using the Megalign program of DNASTar using the Clustal W method.

(f) Structure of residues P461-E706 (panel e) of the human p97 homohexamer viewed from the C-terminus and colored to illustrate the potential impact of pex6 mutations. Alternate p97 subunits are depicted in navy (PEX6) or gray (PEX1) to depict the predicted arrangement of a PEX1-PEX6 heterohexamer. Three subunits are shown as backbone ribbons, and three subunits are shown with surface representations. p97 residues at the positions of Arabidopsis pex6 missense alleles are shown in aqua with side-chains and backbone atoms in spherical representations. Walker A (WA), Walker B (WB), and arginine fingers (asterisks) residue backbones are in green. Co-crystalized ATPγS is in black. The illustration was generated using UCSF Chimera software (Pettersen et al., 2004) from the p97 structure deposited as PDB ID5C18 (Hanzelmann and Schindelin, 2017).
Figure 2. Identification of pex26-1
(a) pex26-1 is IBA resistant. Bars indicate mean hypocotyl lengths of dark-grown seedlings (n ≥ 20). Error bars indicate SD.
(b) Recombination mapping localized the HR127 (pex26-1) mutation on chromosome 3. Numbers indicate recombinant chromosomes over chromosomes assessed at the indicated markers.
(c) PEX26 gene diagram showing locations of pex26 mutations. The pex26-1 G-to-A transition in the nucleotide prior to exon 5 is predicted to disrupt splicing.
(d) PEX26 protein schematic with locations of the predicted transmembrane domain (navy), the *dayu* (Li *et al.*, 2014) and *apem9* (Goto *et al.*, 2011) insertion alleles (triangles), the *apem9-1* missense allele (Goto *et al.*, 2011), and the predicted pex26-1 truncation.  
(e) Alignment of PEX26 homologs from various plants: *Arabidopsis thaliana* (At), *Arabidopsis lyrata*, poplar (*Populus trichocarpa*), rice (*Oryza sativa*), the grass *Brachypodium distachyon*, and spruce (*Picea sitchensis*) highlighting the predicted transmembrane (TM) domain and the *pex26* mutations. The alignment was generated using the Megalign program of DNAStar using the Clustal W method.
Figure 3. *pex6-3* and *pex6-4* defects are complemented by *HA-PEX6* expression; *pex26-1* defects are complemented by *HA-PEX26* expression

(a–b) *pex6-3*, *pex6-4*, and *pex26-1* seedlings are resistant to the inhibitory effects of IBA on hypocotyl and root elongation, partially dependent on exogenous sucrose for hypocotyl elongation, and rescued by *HA-PEX6* or *HA-PEX26* expression. Two complementation lines (+) are shown for each new allele. Bars indicate mean hypocotyl lengths (a; n ≥ 14; except *pex6-4* for which n = 4 on IBA plate due to reduced germination) or mean root lengths (b; n = 16). Error bars indicate SD; different letters above bars indicate significant differences (one-way ANOVA, *P* < 0.001).

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(c–f) pex6 and pex26 seedlings display defects in processing PTS2 proteins and have reduced PEX5 levels that are partially restored by proteasome inhibition. Immunoblots of extracts from 8-d-old seedlings (c), 4- and 6-d-old seedlings (d), rosette leaves from 35-d-old plants (e), or 4-d-old light-grown seedlings treated with or without 50 µM MG132 for 24 h in darkness (f) were serially probed with antibodies to the indicated proteins. The positions of molecular mass markers (in kDa) are indicated on the right. For thiolase and PMDH, ‘p’ indicates precursor and ‘m’ indicates mature (processed) protein. HSC70 is a loading control. In panel c, asterisks indicate HA-PEX6 degradation products. Graphs below immunoblots indicate the PEX5/HSC70 ratio normalized to the ratio in Wt (c), Wt at 4 or 6 d (d), or mock-treated seedlings (f) from at least three biological replicates including the one shown. Error bars indicate SD.
Figure 4. *pex6* and *pex26* mutants show varying developmental delays

(a–b) Like *pex6-1, pex6-3, pex6-4*, and *pex26-1* seedlings are smaller than wild type, and *pex6-3* and *pex6-4* seedlings are paler green than wild type. Seedlings were grown on sucrose-containing medium in the light for 1 (a) or 2 (b) weeks prior to photography. Scale bar is 1 cm.

(c) Like *pex6-1*, mature *pex6-3* plants are smaller than wild type. Plants were transferred to soil after 2 weeks on sucrose-containing medium and photographed after four additional weeks in light. Scale bar is 5 cm.
Figure 5. Ubiquitination-related peroxin mutations can exacerbate or ameliorate pex26-1 defects
(a–b) pex26-1 growth defects on media lacking sucrose are exacerbated (pex4-1, pex10-2, pex12-1) or ameliorated (pex2-1) by ubiquitination-related peroxin mutants (a), and pex26-1 double mutants remain resistant to the inhibitory effects of IBA on hypocotyl (a) and root elongation (b). Bars indicate mean hypocotyl lengths (a) or mean root lengths (b) (n ≥ 13). Error bars indicate SD; different letters above bars indicate significant differences (one-way ANOVA, P < 0.001).
(c–e) Low PEX5 levels in pex26-1 seedlings are restored by pex4-1, pex2-1, pex10-2, and pex12-1 mutants, and PTS2-processing defects are exacerbated by a subset of these mutants. Seedling immunoblots were serially probed with antibodies to the indicated proteins. An asterisk in e indicates a cross-reacting band. Graphs below immunoblots indicate PEX5/Gonzalez et al. Page 30
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HSC70 ratios normalized to the ratios in Wt averaged from three biological replicates including the one shown. Error bars indicate SD.

(f) *pex26-1* increases PEX10 levels in *pex10-2* but not in *pex2-1*. An immunoblot was probed with the indicated antibodies. The graph below the immunoblots indicates the PEX10/HSC70 ratio normalized to the ratio in Wt averaged from three biological replicates including the one shown. Error bars indicate SD.
**Figure 6. PEX5 is predominantly membrane-associated in pex6 mutants**

Homogenates (H) prepared from seedlings grown for 1 d in light followed by 5 d darkness were separated by centrifugation to isolate an organellar pellet (P) and cytosolic supernatant (S). Fractions were subjected to immunoblotting with the indicated antibodies. HSC70 and PEX7 are predominantly soluble, whereas PEX14 and the mitochondrial (mito) ATP synthase subunit α localize in the organelle fractions. The graph indicates the mean ratio of PEX5 recovered in the supernatant or pellet compared to the amount of PEX5 in the homogenate for that genotype in three biological replicates including the one shown. Error bars indicate SD.
Figure 7. Overexpressing PEX5 exacerbates or ameliorates pex6 and pex26 defects
(a–b) Overexpressing PEX5 improves growth of dark-grown pex6-1 and pex6-3 seedlings and exacerbates pex26-1 defects (a) without markedly altering IBA responses of light-grown seedlings (b). Plant lines with (+) and without (−) 35S:PEX5 were grown on the indicated media. Bars indicate mean hypocotyl lengths (a; n ≥ 14) or root lengths (b; n = 16). Error bars indicate SD; different letters above bars indicate significant differences (one-way ANOVA, P < 0.001).
(c–d) Overexpressing *PEX5* worsens *pex6-4* and *pex26-1* PTS2-processing defects. Immunoblots of extracts from dark-grown (c) and light-grown (d) seedlings were serially probed with antibodies to the indicated proteins.

(e) Overexpressing *PEX5* prevents oil body persistence in *pex6-1* and *pex6-3*, but not *pex6-4* or *pex26-1*. Confocal images of cotyledon cells without (top two rows) or with (bottom two rows) the 35S:*PEX5* construct are shown. Scale bar is 20 µm. Samples were excited using a 543-nm Helium Neon laser and Nile red emission was collected at 530-626 nm using a 47.1 µm pinhole to image 0.8 µm optical sections.
Figure 8. Oil bodies persist in *pex6* and *pex26* mutants, which display varying peroxisomal import defects
(a–e) Confocal images of cotyledon epidermal (a, b, c), mesophyll (d), and hypocotyl (e) cells of 5-d-old seedlings are shown. Panel c shows digitally magnified region of epidermal cells boxed in panel b that includes peroxisome-oil body clusters in *pex6-1, pex6-3, pex6-4, and pex26-1*. Cotyledons were stained with Nile red and fluorescence was excited using a 488-nm Argon laser. Emissions were collected at 493-526 for GFP (green) and 587-643 for Nile red (magenta) using a 71.7 µm pinhole to image 1.3 µm optical sections.
Figure 9. Working model for *Arabidopsis* PEX5 recycling illustrating potential effects of *pex6* (and *pex26*) mutations

(a) In wild type, cytosolic PEX5 (green outline) delivers matrix protein cargo (yellow), which promotes peroxisome function. After cargo delivery, PEX5 in the membrane (red outline) is mono-ubiquitinated (Ub) and extracted by the PEX1-PEX6-PEX26 ATPase complex, preventing extensive PEX5 poly-ubiquitination. Numbered shapes represent peroxins. Peroxisomal ubiquitin-protein ligases are postulated to catalyze PEX5 mono-ubiquitination (PEX12 and PEX10) or poly-ubiquitination (PEX2 and PEX10) in *Arabidopsis* as has been shown in yeast (Platta et al., 2009; El Magraoui et al., 2012). Although different ubiquitin-conjugating enzymes promote mono- and poly-ubiquitination in yeast (reviewed in Platta et al., 2014), the ability of a *pex4* mutant to suppress decreased PEX5 levels in *pex6-1* (Ratzel et al., 2011) suggests that PEX4 promotes both processes in *Arabidopsis*.

(b) When PEX6 or PEX26 function is reduced, PEX5 recycling slows, and PEX5 accumulates in the peroxisomal membrane to the detriment of peroxisome function. This lingering PEX5 is subject to poly-ubiquitination, which may trigger pexophagy of the entire organelle, further decreasing peroxisome function. Alternatively, poly-ubiquitinated PEX5 may be extracted from the peroxisomal membrane by the PEX1-PEX6-PEX26 complex for proteasomal degradation. *pex4-1, pex2-1, pex10-2,* or *pex12-1* mutations increase PEX5 levels in the *pex26-1* mutant, but only *pex2-1* restores peroxisome function, suggesting that slowing PEX5 poly-ubiquitination allows additional recycling. Overexpressing PEX5 rescues some defects of *pex6-1* and *pex6-3* while exacerbating *pex6-4* and *pex26-1* defects, consistent with the possibility that these mutants differentially impair the ability of the...
ATPase complex to extract mono-ubiquitinated PEX5 for recycling versus poly-ubiquitinated PEX5 for degradation.