Short title:
Plant PEX1 is essential for peroxisomes

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The PEX1 ATPase stabilizes PEX6 and plays essential roles in peroxisome biology

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One sentence summary:
Novel missense alleles of the Arabidopsis PEX1 ATPase reveal essential peroxisomal roles that impact embryogenesis and plant growth.

Author contributions:
M.A.R., W.A.F., K.L.G., and B.B. conceived and planned the experiments. M.A.R. performed most of the experiments and wrote the first draft of the manuscript. W.A.F. organized the screen that isolated pex1-2, analyzed pex1-2 genomic sequencing data, and obtained pex1-2 plants expressing GFP-PTS1. K.L.G. constructed plasmids for overexpressing PEX1 and PEX6. J.P. mapped pex1-3 and assisted with mutant characterization. M.J.V. isolated pex1-2 and conducted pex1-2 recombination mapping. A.B.P. assisted with mutant characterization. All authors revised the manuscript and approved the final version.
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ABSTRACT

A variety of metabolic pathways are sequestered in peroxisomes, conserved organelles that are essential for human and plant survival. Peroxin (PEX) proteins generate and maintain peroxisomes. The PEX1 ATPase facilitates recycling of the peroxisome matrix protein receptor PEX5 and is the most commonly affected peroxin in human peroxisome biogenesis disorders.

Here we describe the isolation and characterization of the first reported Arabidopsis pex1 missense alleles: pex1-2 and pex1-3. pex1-2 displayed peroxisome-related defects accompanied by reduced PEX1 and PEX6 levels. These pex1-2 defects were exacerbated by growth at high temperature and ameliorated by growth at low temperature or by PEX6 overexpression, suggesting that PEX1 enhances PEX6 stability and vice versa. pex1-3 conferred embryo lethality when homozygous, confirming that PEX1, like several other Arabidopsis peroxins, is essential for embryogenesis. pex1-3 displayed symptoms of peroxisome dysfunction when heterozygous; this semi-dominance is consistent with PEX1 forming a hetero-oligomer with PEX6 that is poisoned by pex1-3 subunits. Blocking autophagy partially rescued PEX1/pex1-3 defects, including restoration of normal peroxisome size, suggesting that increasing peroxisome abundance can compensate for the deficiencies caused by pex1-3, and that the enlarged peroxisomes visible in PEX1/pex1-3 may represent autophagy intermediates. Overexpressing PEX1 in wild-type plants impaired growth, suggesting that excessive PEX1 can be detrimental.

Our genetic, molecular, and physiological data support the heterohexamer model of PEX1-PEX6 function in plants.
INTRODUCTION

Critical steps of vital metabolic pathways are housed in single lipid bilayer-bound organelles known as peroxisomes. For example, plant peroxisomes house enzymes for reactive oxygen species detoxification, photorespiration, the glyoxylate cycle, and β-oxidation (reviewed in Hu et al., 2012; Reumann and Bartel, 2016). Proteins necessary for peroxisome biogenesis and maintenance are termed peroxins (PEX proteins), and most known peroxins facilitate protein import into the peroxisome matrix from their site of synthesis in the cytosol. Matrix proteins generally have a peroxisomal targeting signal (PTS) 1 or 2 (Gould et al., 1989; Swinkels et al., 1991), which is recognized in the cytosol by receptors PEX5 (McCollum et al., 1993) or PEX7 (Marzioch et al., 1994), respectively. The binding of PEX7 to PEX5 in plants (Nito et al., 2002) and mammals (Braverman et al., 1998) couples import of PTS2 and PTS1 proteins (Hayashi et al., 2005; Woodward and Bartel, 2005; Khan and Zolman, 2010; Ramón and Bartel, 2010). The actual transport step is not fully elucidated, but in yeast, PEX5 docks at the PEX14 peroxisomal membrane protein (Hayashi et al., 2000; Stanley and Wilmanns, 2006), where PEX5 membrane insertion allows cargo delivery into the peroxisomal matrix (Meinecke et al., 2010). Once in the peroxisome, the N-terminal region containing the PTS2 signal is cleaved in plants by the protease DEG15 (Helm et al., 2007). After cargo delivery, yeast PEX5 is mono- or diubiquitinated (Kragt et al., 2005; Williams et al., 2007) to signal for retrotranslocation to the cytosol by PEX1 and PEX6 (Platta et al., 2005), allowing for PEX5 reuse (Dammai and Subramani, 2001). Alternatively, polyubiquitinated PEX5 can be degraded by the proteasome (reviewed in Platta and Erdmann, 2007). Although PEX5 ubiquitination has not been directly demonstrated in plants, analysis of PEX5 levels and localization in Arabidopsis mutants defective in the peroxisomal ubiquitination machinery implicate similar pathways for recycling or degrading plant PEX5 (Mano et al., 2006; Ratzel et al., 2011; Kao et al., 2016).

PEX1 and PEX6 are the only members of the AAA family of ATPases Associated with diverse cellular Activities (reviewed in Patel and Latterich, 1998) implicated in peroxisome biogenesis and are closely related to p97, which functions in ER-associated protein degradation to retrotranslocate ER proteins to the cytosol (Ye et al., 2001). PEX1 and PEX6 have two ATPase domains, named D1 and D2 (Fig. 1D). Each ATPase domain usually contains Walker A and Walker B motifs needed for nucleotide binding and hydrolysis, respectively (reviewed in Grimm et al., 2012), and a second region of homology (SRH) domain needed for ATPase activity (Karata et al., 1999). In humans, mutations in PEX1 are the most common cause of peroxisome biogenesis disorders, a spectrum of generally fatal conditions in which peroxisomes are not formed or inefficiently import matrix proteins (reviewed in Waterham and Ebberink, 2012). Similarly, yeast null pex1 mutants lack functional peroxisomes (Erdmann et al., 1989),
and defects in the yeast PEX1 D2 lead to empty vesicles rather than functional peroxisomes (Birschmann et al., 2005).

In yeast, PEX1 and PEX6 form a heterohexamer (Saffian et al., 2012) composed of three units of each peroxin arranged in an alternating ring with a central pore (Gardner et al., 2015) through which the complex might pull PEX5 (Ciniawsky et al., 2015). PEX1-PEX6 interaction in yeast and humans requires the PEX1 D2 and D1 regions (Birschmann et al., 2005; Tamura et al., 2006). In addition to supporting heterohexamer assembly, ATP hydrolysis elicits conformational changes in the PEX1-PEX6 complex that facilitate retrotranslocation of ubiquitinated PEX5 in yeast (reviewed in Platta et al., 2016) and humans (Tamura et al., 2006). PEX5 retrotranslocation requires ATP hydrolysis by both PEX1 and PEX6 D2 regions (Platta et al., 2005; Tamura et al., 2006). Defects in PEX1 or PEX6 can lead to inefficient PEX5 retrotranslocation from the peroxisomal membrane accompanied by PEX5 polyubiquitination and degradation (reviewed in Platta et al., 2016). Saccharomyces cerevisiae pex1 mutants accumulate polyubiquitinated PEX5 (Kragt et al., 2005), and pex1 mutants in humans (Dodt and Gould, 1996) and Pichia pastoris (Collins et al., 2000) have reduced PEX5 levels.

The PEX6 N-terminal region recruits the PEX1-PEX6 heterohexamer to the peroxisome by interacting with the cytosolic domain of a tail-anchored peroxisomal membrane protein: PEX15 in yeast (Birschmann et al., 2003), PEX26 in humans (Matsumoto et al., 2003), and PEX26, also known as ABERRANT PEROXISOME MORPHOLOGY9 or DAYU (Goto et al., 2011; Li et al., 2014), in Arabidopsis. Human PEX26 also binds to PEX14 and dissociates upon PEX1-PEX6 ATPase activity (Tamura et al., 2014). Because PEX14 recruits PEX5 to the peroxisome, this finding is consistent with the hypothesis that PEX26 positions PEX1-PEX6 in proximity to PEX14-bound PEX5 to facilitate PEX5 retrotranslocation to the cytosol.

Peroxisomes perform vital functions in plants. Arabidopsis is an oilseed plant that catabolizes triacylglycerol stored in the seed to support early seedling growth. Because peroxisomes are the sole site of fatty acid β-oxidation in plants (reviewed in Graham, 2008), pex mutants can display reduced growth that is ameliorated by supplementation with an alternative fixed carbon source such as sucrose (reviewed in Bartel et al., 2014). Because peroxisomes also house the β-oxidation of the auxin precursor IBA to the active auxin indole-3-acetic acid (Zolman et al., 2000; Strader et al., 2010), screening for IBA resistance can yield mutants defective in peroxisome biogenesis (reviewed in Bartel et al., 2014). Such mutants are often also resistant to the synthetic IBA analog 2,4-dichlorophenoxybutyric acid (2,4-DB), which is similarly β-oxidized to the auxinic herbicide 2,4-dichlorophenoxyacetic acid (Wain and Wightman, 1954; Hayashi et al., 1998). At a cellular level, defects in peroxisomal size, positioning, and matrix protein import can be visualized by tagging GFP with a PTS, which also has formed the basis of screens for mutants with peroxisome defects (Mano et al., 2006; Burkhart et al., 2013; Rinaldi et
Because PTS2 cleavage occurs after import, mutations in peroxin genes often impair PTS2 processing (reviewed in Bartel et al., 2014). The PTS2-processing protease DEG15 is a PTS1 protein (Helm et al., 2007), and so PTS2-processing defects can reflect PTS1 and/or PTS2 import defects.

Like in yeast and mammals, *Arabidopsis* PEX6 binds PEX26, and PEX1 localization to peroxisomes requires both PEX6 and PEX26 (Goto et al., 2011). *Arabidopsis* pex6-1 carries a missense mutation in the D2 and has reduced PEX5 levels (Zolman and Bartel, 2004) due to proteasomal PEX5 degradation (Kao and Bartel, 2015), and PEX5 overexpression partially rescues pex6-1 defects (Zolman and Bartel, 2004; Burkhart et al., 2013), suggesting that reduced PEX5 function contributes to the defects of some plant *pex* mutants. *PEX1* expression increases under starvation and senescence conditions (Charlton et al., 2005) and following wounding or hydrogen peroxide treatment (Lopez-Huertas et al., 2000), suggesting a role for *PEX1* in oxidative stress responses. An RNAi knockdown line targeting *PEX1* shows peroxisome-related physiological defects (Nito et al., 2007). A reverse-genetic screen for *EMBRYO-DEFECTIVE* (*EMB*) genes identified two insertional mutations in *PEX1* (*emb2817-1* and *emb2817-2*) that confer embryos arresting at the preglobular stage (http://www.seedgenes.org/; Muralla et al., 2011).

Although mutants in almost all predicted single-gene peroxins have been isolated in forward-genetic screens for peroxisomal defects in *Arabidopsis* (reviewed in Bartel et al., 2014), viable *pex1* mutants have not been reported in plants. Here, we describe two *Arabidopsis* mutants in *PEX1*, *pex1-2* and *pex1-3*, that display distinct peroxisome-related defects and harbor missense mutations in the region encoding the PEX1 D2. We found that PEX1 contributed to PEX6 accumulation and peroxisomal matrix protein import, and that excessive PEX1 caused growth defects in wild type. The *pex1-3* mutation could not be recovered as a homozygote, confirming that PEX1 is necessary for embryogenesis, and was semi-dominant, consistent with the heterohexamer model of PEX1-PEX6 function.

**RESULTS**

*pex1* mutants were isolated from screens for peroxisomal defects

Two *pex1* mutants emerged from different screens for *Arabidopsis* seedlings with peroxisomal defects. We isolated *pex1-2* from a screen for IBA resistance in dark-grown seedlings (Strader et al., 2011; Kao et al., 2016). Dark-grown *pex1-2* seedlings displayed longer hypocotyls than wild type on inhibitory concentrations of IBA (Fig. 1A). Whole-genome sequencing of pooled backcrossed lines revealed several mutations including *pex1-2* (Supplemental Fig. 1A; Supplemental Data Set 1). This C6606-to-T changed Leu993 to Phe in
the D2 SRH (Fig. 1C and D). The analogous residue is conserved as an Ile or Leu in human PEX1, Arabidopsis and human PEX6, and mouse p97 (Fig. 1D).

We isolated pex1-3 in a screen for seedlings displaying a pattern of peroxisome-targeted GFP (GFP-PTS1) distribution that differed from wild type (Rinaldi et al., 2016). The pex1-3 mutant displayed larger GFP-PTS1 puncta than wild type combined with GFP-PTS1 mislocalized to the cytosol (Fig. 1B). Recombination mapping suggested a causal heterozygous mutation at the top of chromosome 5 (Fig. 1C). Whole-genome sequencing of two backcrossed lines revealed a heterozygous pex1-3 mutation (Supplemental Fig. 1B; Supplemental Data Set 1) that changed Gly974 to Glu. Gly974 is in the D2 intersubunit signaling motif (Augustin et al., 2009) and is conserved in other AAA proteins, including human PEX1, Arabidopsis and human PEX6, and mouse p97 (Fig. 1D).

pex1-3 is semi-dominant and confers embryo lethality when homozygous

The peroxisomal defects of pex1-3 (Fig. 1B) segregated in every generation, and we were unable to obtain a homogeneous population. This persistent heterogeneity suggested a semi-dominant causal mutation that conferred lethality when homozygous. Indeed, our recombination mapping (Fig. 1C) and whole genome sequencing (Supplemental Fig. 1B) both revealed heterozygosity at the top of chromosome 5, consistent with a lethal mutation in this region. Adult PEX1/pex1-3 plants resembled wild type in overall morphology (Fig. 2A). However, when we examined seed development in PEX1/pex1-3 plants, we found empty or missing seeds in green seedpods (Fig. 2B) and shriveled seeds among plump healthy seeds in mature seedpods (Fig. 2C and D), consistent with the possibility that pex1-3/pex1-3 homozygous embryos arrest and die during development. Moreover, 1188 individual progeny of PEX1/pex1-3 heterozygotes were genotyped in the course of this research: 431 were wild type, 757 were PEX1/pex1-3 heterozygotes, and none were pex1-3/pex1-3 homozygotes. This 1:1:9:0 ratio resembles the 1:2:0 ratio expected for Mendelian segregation of a homozygous-lethal mutation. We therefore characterized the effects of the pex1-3 mutation as a heterozygote.

Mutations in PEX1 lead to peroxisomal defects

We compared our pex1 mutants to pex6-1, a mutant defective in the PEX1-interacting ATPase (Zolman and Bartel, 2004; Goto et al., 2011), using various assays that monitor peroxisome function. Like PEX1/pex1-3, adult pex1-2 plants generally resembled wild type (Fig. 2A) whereas pex6-1 is a pale green dwarf plant (Fig. 2A; Zolman and Bartel, 2004). pex1-2 and PEX1/pex1-3 were partially resistant to the inhibitory effects of 2,4-DB on hypocotyl and root elongation (Fig. 2E and F), somewhat resistant to the inhibitory effects of IBA on hypocotyl elongation in the dark (Fig. 2E), but less noticeably resistant to the inhibitory effects of IBA on...
root elongation in the light (Fig. 2F). In contrast, pex6-1 roots and hypocotyls are clearly resistant to both precursors (Fig. E and F; Zolman and Bartel, 2004; Burkhart et al., 2013). Similarly, pex1-2 and PEX1/pex1-3 displayed only partial resistance to the promotion of lateral roots by IBA (Fig. 2G), compared to the complete resistance of pex6-1 in this assay (Zolman and Bartel, 2004). This IBA resistance was not due to a general auxin-response defect, as the mutants produced lateral roots like wild type in response to the auxin analog 1-napthaleneacetic acid (NAA; Fig. 2G), which does not require β-oxidation for activity.

pex1 mutants did not display consistent growth defects attributable to reduced fatty acid mobilization. Unlike pex6-1, pex1-2 and PEX1/pex1-3 resembled wild type when grown on medium without sucrose (Fig. 2E and F). Together, both physiological assays (Fig. 2E-G) and adult morphology (Fig. 2A) indicated that peroxisome function was less disrupted in pex1-2 and PEX1/pex1-3 than in the pex6-1 mutant.

Along with physiological defects, both pex1 mutants displayed defects in matrix protein import. pex6-1 shows substantial GFP-PTS1 mislocalization to the cytosol and few GFP-PTS1 puncta (Fig. 3A; Burkhart et al., 2014), indicating a strong defect in peroxisomal matrix protein import. Similarly, PEX1/pex1-3 seedlings showed both cytosolic GFP-PTS1 localization and GFP-PTS1 puncta, some of which were larger than wild-type puncta (Fig. 1B and 3A). In contrast, pex1-2 was variable in this assay; some 8-d-old seedlings appeared similar to wild type whereas others showed GFP-PTS1 mislocalization to the cytosol along with GFP-PTS1 puncta (Fig. 3A).

In a second assay to monitor matrix protein import, we examined PTS2 processing in the mutants. Whereas pex6-1 has a notable PTS2-processing defect (Fig. 3B; Zolman et al., 2005), PEX1/pex1-3 presented only a slight PTS2-processing defect in young seedlings (Fig. 3B), and pex1-2 displayed a slight PTS2-processing defect in older plants (Fig. 3B).

**PEX1 overexpression complements pex1 mutants**

To confirm that the mutations we identified in PEX1 were responsible for the observed phenotypes, we performed a complementation assay by crossing pex1-2/pex1-2 and PEX1/pex1-3. The pex1-2/pex1-3 transheterozygote was underrepresented in the resultant F1; 37 individuals were pex1-2/pex1-3 whereas 79 were PEX1/pex1-2. This 1:2 ratio deviated from the expected 1:1 ratio, suggesting that some pex1-2/pex1-3 individuals died during embryogenesis. Furthermore, of the 37 pex1-2/pex1-3 transheterozygous individuals recovered, 24 barely germinated and did not develop into seedlings. Among the 13 transheterozygous pex1-2/pex1-3 seedlings that emerged from the seed coat, the two on a plate containing 2,4-DB had elongated hypocotyls (Fig. 4A), also suggesting failure to complement. The pex1-2/pex1-3 transheterozygotes that
developed into seedlings did not survive to adulthood. This failure of pex1-2 to complement the lethality of pex1-3 suggested that both identified pex1 mutations were causal.

We also introduced constructs that used the cauliflower mosaic virus 35S promoter to express untagged or HA-tagged PEX1 cDNAs into the pex1 mutants and tested for complementation. HA-PEX1 expression in pex1-2 restored IBA sensitivity in lateral root promotion (Fig. 4B and C) and improved PTS2 processing (Fig. 4D), again indicating that the pex1-2 mutation was causal. Moreover, we were able to obtain homozygous pex1-3/pex1-3 lines overexpressing PEX1. These lines produced viable offspring, indicating that the pex1-3 mutation was responsible for the lethality. pex1-3 35S:PEX1 homozygotes were smaller than wild type as seedlings (Fig. 4F) and adults (Fig. 4G) and still displayed peroxisomal defects, including IBA resistance in lateral root production (Fig. 4B and C) and incomplete PTS2 processing (Fig. 4E). These defects may persist because levels of wild-type PEX1 are insufficient to counteract the defective pex1-3 protein that remains in these plants or because PEX1 is overexpressed to detrimental levels.

We transformed wild-type plants with constructs overexpressing PEX1 or PEX6 in parallel. Transformants for 35S:HA-PEX6 were easy to obtain and resembled wild type in growth (Fig. 4H) whereas transformants for 35S:HA-PEX1 were fewer than 35S:HA-PEX6 transformants and smaller than wild type, suggesting that PEX1 overexpression is deleterious. To directly compare the consequences of PEX1 overexpression in wild type and pex1-2, we crossed a pex1-2 line rescued with 35S:HA-PEX1 (Fig. 4B-D) to wild type and isolated the transgene in a wild-type PEX1 background. Interestingly, pex1-2 carrying 35S:HA-PEX1 was morphologically more similar to wild type than wild type with 35S:HA-PEX1 (Fig. 4H). Thus the HA-PEX1 levels produced by this transformation event are sufficient to restore PEX1 function in pex1-2 but appear to cause growth defects in a wild-type background, suggesting that excessive PEX1, or PEX1 expressed ectopically in tissues normally lacking PEX1, is deleterious. Consistent with this hypothesis, we found that PEX1 and HA-PEX1 levels were only modestly increased compared to endogenous PEX1 levels (Fig. 4D and E) both in wild type and in lines that restore viability to pex1-3 and IBA responsiveness and PTS2 processing to pex1-2.

**PEX5 overexpression fails to rescue pex1 mutants**

PEX5 overexpression partially rescues pex6-1 defects in growth, sucrose dependence, and PTS2-processing (Zolman and Bartel, 2004; Burkhart et al., 2013), presumably because PEX5 is excessively organelle-associated (Ratzel et al., 2011) and degraded by the proteasome in pex6-1 (Kao and Bartel, 2015). Unlike in pex6-1, PEX5 levels generally resembled wild-type levels in PEX1/pex1-3 (Fig. 3B, 4E, 5B and C) and we did not find consistently low PEX5 levels in pex1-2 (Fig. 3B, 5B and C). Also unlike pex6-1 seedlings, PEX5 was distributed in organellar and
cytosolic fractions similarly to wild type in extracts from pex1-2 and PEX1/pex1-3 progeny (Supplemental Fig. 2). Furthermore, overexpressing PEX5 in pex1-2 or PEX1/pex1-3 did not improve IBA responsiveness (Fig. 5A) or PTS2 processing (Fig. 5B and C). In fact, PEX5 overexpression worsened the PTS2-processing defect of pex1-2 (Fig. 5C).

pex1-2 protein accumulation and pex1-2 peroxisomal function are reduced at elevated growth temperature

We noticed that older pex1-2 seedlings accumulated less PEX1 protein than wild type or the PEX1/pex1-3 heterozygote (Fig. 4D). To further investigate the connection between PEX1 protein levels and peroxisomal defects, we examined peroxisome-related phenotypes following growth at temperatures higher (28 °C) or lower (15 °C) than our standard growth temperature (22 °C), anticipating that higher temperatures might exacerbate pex1-2 defects if they were caused by pex1-2 protein misfolding and consequent degradation.

Growth at mildly elevated temperature increases wild-type hypocotyl elongation (Gray et al., 1998), root elongation (Rogg et al., 2001), and lateral root production (Wang et al., 2016). We found that growth at 28 °C increased wild-type root elongation (Fig. 6A) and IBA-responsive lateral root production (Fig. 6B). pex6-1 was resistant to IBA (Fig. 6A and B) and displayed GFP-PTS1 import defects (Fig. 6D) and impaired PTS2 processing (Fig. 6C) at all growth temperatures assayed, although PTS2-processing defects were more apparent at lower temperatures (Fig. 6C), perhaps due to deleterious accumulation of PEX5 in the peroxisomal membrane (Kao and Bartel, 2015). PEX1/pex1-3 generally responded to temperature similarly to wild type (Fig. 6A and B), and cytosolic mislocalization of GFP-PTS1 in PEX1/pex1-3 seedlings was apparent at all three growth temperatures (Fig. 6D), suggesting that the defects in this mutant are not related to PEX1-PEX6 protein levels. In fact, lower temperature appeared to worsen rather than ameliorate the PTS2-processing defect in PEX1/pex1-3 (Fig. 6C).

In contrast to PEX1/pex1-3, high temperature exacerbated pex1-2 IBA resistance in hypocotyl elongation (Fig. 6A) and lateral root production (Fig. 6B). Moreover, high temperature worsened and low temperature ameliorated pex1-2 PTS2-processing (Fig. 6C) and GFP-PTS1 import (Fig. 6D). These temperature-dependent defects in peroxisome physiology and import were accompanied by reduced and elevated PEX1 and PEX6 protein levels at high and low temperatures, respectively (Fig. 6C), suggesting that pex1-2 instability leads to PEX6 degradation and peroxisomal defects.

PEX6 overexpression rescues pex1-2 defects

Our observation that PEX6 levels were reduced in pex1-2 but not in PEX1/pex1-3 (Fig. 5B and C and 6C) suggested that PEX6 stability decreases when PEX1 levels decline. To assess
whether decreased PEX6 levels contributed to pex1-2 defects, we expressed HA-PEX6 from the 35S promoter in pex1-2. We found that HA-PEX6 expression restored pex1-2 IBA sensitivity in lateral root promotion (Fig. 7A and B) and PTS2 processing (Fig. 7C). Moreover, HA-PEX6 expression increased pex1-2 protein levels to resemble wild-type PEX1 levels (Fig. 7C). These results suggest that increased PEX6 levels counteract the pex1-2 instability caused by the pex1-2 missense mutation and that reduced PEX6 and/or PEX1 levels contribute to the defects observed in the pex1-2 mutant.

Because HA-PEX6 expression rescued pex1-2 defects, we attempted the reciprocal experiment to test whether PEX1 overexpression could rescue pex6-1. We crossed plants carrying PEX1-overexpressing constructs to pex6-1. However, we were unable to isolate homozygous pex6-1 carrying 35S:PEX1 or 35S:HA-PEX1, suggesting that PEX1 overexpression did not rescue pex6-1 defects.

pex1-2 and pex6-1 defects are synergistic

To further explore the genetic interactions between PEX1 and PEX6, we constructed a pex1-2 pex6-1 double mutant. pex1-2 pex6-1 seedlings were smaller than either single mutant (Fig. 8A) and displayed worsened PTS2-processing defects (Fig. 8B). The pex1-2 pex6-1 double mutants died as seedlings without leaving progeny, indicating that the peroxisomal defects of pex1-2 and pex6-1 were synergistic.

We also attempted to isolate pex1-3 pex6-1 double mutants. Among progeny from a PEX1/pex1-3 PEX6/pex6-1 heterozygote, we found one PEX1/pex1-3 pex6-1/pex6-1 individual and one pex1-3/pex1-3 pex6-1/pex6-1 individual that were both non-germinated seeds. However, we were unable to isolate PEX1/pex1-3 pex6-1/pex6-1 or pex1-3/pex1-3 pex6-1/pex6-1 seedlings. This lethality suggests that peroxisomal defects of pex1-3 and pex6-1 also were synergistic.

Blocking autophagy partially rescues PEX1/pex1-3 defects

The enlarged GFP-PTS1 puncta observed in PEX1/pex1-3 (Fig. 1B, 3A, and 6D) were evocative of the enlarged peroxisome-targeted GFP puncta observed when the peroxisomal protease LON2 is dysfunctional (Farmer et al., 2013). Because preventing autophagy can restore peroxisomal functions to lon2 mutants, we combined the PEX1/pex1-3 mutation with atg7-3, a null mutation in an essential autophagy component (Doelling et al., 2002). We found that preventing autophagy rescued PEX1/pex1-3 IBA responsiveness (Fig. 9A) and GFP-PTS1 puncta size (Fig. 9B). Interestingly, these phenotypic ameliorations were not accompanied by notable improvements in PTS1 import (Fig. 9B) or PTS2 processing (Fig. 9C). These results indicate that intact autophagy machinery is required to develop enlarged GFP-PTS1 puncta in
PEX1/pex1-3 but that the defects in matrix protein import in this mutant do not result from excessive autophagy of peroxisomes.

DISCUSSION

PEX1 is essential for embryogenesis and contributes to peroxisome function in plants

PEX1 was among the first peroxins identified in yeast and mammals. However, nearly two decades after the first reports of Arabidopsis peroxin mutants (Hayashi et al., 1998; Zolman et al., 2000), viable mutants have been reported for all single-copy peroxin genes except for PEX1 (reviewed in Bartel et al., 2014). This deficit is perhaps because null mutations in peroxins can be lethal in plants. In fact, PEX14 is the only Arabidopsis peroxin for which reported null alleles are viable (Monroe-Augustus et al., 2011). In contrast, lethality results from eliminating PEX26, the peroxisomal tether of the PEX1-PEX6 heterohexamer (Goto et al., 2011; Li et al., 2014), PEX13, which aids in docking of cargo-loaded PEX5-PEX7 to the peroxisome (Boisson-Dernier et al., 2008; Woodward et al., 2014), or any of the three peroxins implicated in PEX5 ubiquitination: PEX2 (Hu et al., 2002), PEX10 (Schumann et al., 2003; Sparkes et al., 2003), and PEX12 (Fan et al., 2005). In agreement with previous reports (http://www.seedgenes.org/; Muralla et al., 2011), we found that PEX1 is also essential in Arabidopsis; a homozygous pex1-3 missense mutation conferred lethality (Fig. 2B-D) that was rescued by PEX1 overexpression (Fig. 4F and G).

The pex1-2 and pex1-3 mutations both altered conserved residues in D2, the second AAA domain (Fig. 1D). An Ile989-to-Thr mutation in the human PEX1 residue that analogous to the Leu993 residue mutated in pex1-2 (Fig. 1D) results in peroxisome biogenesis disorders when compounded with other PEX1 mutations (Maxwell et al., 2005; Smith et al., 2016). PEX1 D2 mutations can confer general peroxisomal defects (Birschmann et al., 2005) and decrease ATPase activity (Gardner et al., 2015) and PEX5 export in yeast (Platta et al., 2005), reduce PEX1 binding to PEX6 in yeast (Birschmann et al., 2005) and humans (Tamura et al., 2006), and impair PEX26 disassociation from PEX14 in humans (Tamura et al., 2014). The peroxisomal defects found in our two Arabidopsis PEX1 D2 mutants indicate that this domain is also important for peroxisome function in plants.

In contrast to the marked growth defects of a PEX1 RNAi line (Nito et al., 2007) and pex6-1 seedlings (Zolman and Bartel, 2004), pex1-2 and PEX1/pex1-3 seedlings grew similarly to wild type even on medium lacking sucrose (Fig. 2E and F), indicating that sufficient PEX1 function remains in these mutants to metabolize enough fatty acids to achieve robust seedling growth. pex1-2 defects became more apparent with age; young pex1-2 seedlings displayed slight or no PTS2-processing defects (Fig. 3B, 5B, and 8B), whereas older pex1-2 plants displayed clear PTS2-processing defects (Fig. 3B, 4D, 5C, and 7C). This age-dependence might explain the
pex1-2 sucrose-independence and variability in GFP-PTS1 import that we observed in pex1-2 seedlings (Fig. 3A), as some seedlings might develop defects more quickly than others.

PEX1 limits autophagy of peroxisomes

Autophagy is a bulk degradation pathway to recycle cell constituents (Li and Vierstra, 2012). Peroxisome-specific autophagy (pexophagy) was recently discovered in plants (reviewed in Young and Bartel, 2015); autophagy-defective seedlings have increased numbers of peroxisomes (Kim et al., 2013; Shibata et al., 2013). We isolated PEX1/pex1-3 from a microscopy-based screen for mutants with altered GFP-PTS1 patterning that were visible under relatively low magnification (Rinaldi et al., 2016). Although this screen yielded mutations in 15 genes that conferred aberrantly large or clustered peroxisomes (Rinaldi et al., 2016), PEX1/pex1-3 was the only peroxin mutant recovered. Because PTS1 import defects are common to numerous pex mutants (reviewed in Bartel et al., 2014) that were not recovered in this screen, we presumably isolated PEX1/pex1-3 because of its enlarged GFP-PTS1 puncta (Fig. 1B and 3A), a phenotype that is not generally reported for pex mutants. The enlarged peroxisomes in PEX1/pex1-3 are reminiscent of pxn peroxisomes (Mano et al., 2011), which are defective in a peroxisomal transporter (Agrimi et al., 2012; Bernhardt et al., 2012; Van Roermund et al., 2016), and lon2 peroxisomes (Farmer et al., 2013; Goto-Yamada et al., 2014), which are defective in a peroxisomal protease needed for sustained peroxisomal function (Lingard and Bartel, 2009). Indeed, four pxn and three lon2 loss-of-function alleles were recovered from the same GFP-PTS1-based screen (Rinaldi et al., 2016) from which we recovered PEX1/pex1-3. The large GFP-PTS1 puncta in pxn mutants are not reduced when autophagy is prevented (Rinaldi et al., 2016), whereas preventing autophagy in lon2 eliminates enlarged puncta (Farmer et al., 2013; Goto-Yamada et al., 2014), indicating that enlarged puncta can be pexophagy intermediates (Farmer et al., 2013; Goto-Yamada et al., 2014). Like in lon2, we found that preventing autophagy in PEX1/pex1-3 eliminated the enlarged puncta (Fig. 9B), suggesting that these PEX1/pex1-3 structures were autophagy intermediates. Preventing autophagy in lon2 restores not only peroxisome size and IBA responsiveness, but also matrix protein import and PTS2 processing (Farmer et al., 2013; Goto-Yamada et al., 2014), indicating that lon2 defects are largely due to heightened pexophagy of otherwise functioning peroxisomes. Like in lon2, PEX1/pex1-3 IBA responsiveness was restored when autophagy was prevented (Fig. 9A). Unlike in lon2, however, atg7-3 did not restore matrix protein import or PTS2 processing in PEX1/pex1-3 seedlings (Fig. 9B and C). These differences suggest that although PEX1 dysfunction triggers pexophagy of peroxisomes that are still capable of IBA-to-IAA conversion, preventing pexophagy is not the sole peroxisomal function of PEX1.
Like in Arabidopsis PEX1/pex1-3, pexophagy is induced in yeast pex1 and pex6 mutants (Nuttall et al., 2014), perhaps because the ubiquitinated substrates that are not efficiently retrotranslocated from the peroxisomal membrane attract the autophagy machinery. Similarly, RNAi-mediated depletion of PEX1 or PEX26 in mammalian cells leads to accumulation of ubiquitinated PEX5 and elevated pexophagy, and autophagy inhibitors improve peroxisome functioning in cells carrying the pex1-G843D missense allele (Law et al., 2017). Thus, preventing pexophagy appears to be a conserved function of the peroxisomal AAA ATPase complex.

**PEX1 and PEX6 are interdependent**

The pex1-3 mutation changes a conserved Gly residue in the PEX1 D2 inter-subunit signaling region to a Glu residue (Fig. 1D). In related ATPases, this region interacts with the neighboring subunit near the ATP-binding site (Augustin et al., 2009). Mutation of the analogous residue (Gly610) in the mammalian AAA homohexamer p97 to an Ala, Val, or Cys reduces p97 ATPase activity by more than 50% (Huang et al., 2012). The similarity between PEX1 and p97 (Fig. 1D) suggests that the pex1-3 substitution likely impairs the ability of PEX1 to appropriately influence the ATPase activity of the neighboring PEX6 subunit.

The pex1-3 mutation conferred lethality when homozygous (Fig. 2B-D) and semi-dominant peroxisome-related defects when heterozygous (Fig. 2E-G and 3A and B). Some human PEX1 mutations also confer mild symptoms even when heterozygous (Majewski et al., 2011). Yeast PEX1 and PEX6 form a heterohexamer with three subunits of each protein (Saffian et al., 2012; Blok et al., 2015; Ciniawsky et al., 2015; Gardner et al., 2015), providing a rationale for semi-dominance of a pex1 mutation. In a PEX1/pex1-3 heterozygote, most (7/8) PEX1-PEX6 heterohexamers would be expected to have at least one pex1-3 unit that might decrease the activity or stability of the complex, a phenomenon known as subunit poisoning.

Interestingly, PEX1/pex1-3 defects became less apparent with age. Eight-d-old PEX1/pex1-3 seedlings were partially IBA resistant in lateral root promotion (Fig. 2G, 4B and C), had a slight PTS2-processing defect (Fig. 3B, 4E, and 5B), and displayed enlarged GFP-PTS1 puncta and cytosolic GFP-PTS1 mislocalization (Fig. 1B and 3A). In contrast, PEX1/pex1-3 displayed normal adult morphology (Fig. 2A and 4G) and fully processed PTS2 proteins in older seedlings (Fig. 5C). Perhaps pex1-3 is degraded over time, allowing hexamers consisting of wild-type PEX1 and PEX6 to accumulate. Although PEX1 overexpression prevented the lethality of homozygous pex1-3 mutants (Fig. 4F and G), peroxisomal defects persisted in pex1-3 35S:PEX1 plants (Fig. 4B, C, and E), presumably because pex1-3 imparts defects even in the presence of wild-type PEX1.
In spite of clear reduction in matrix protein import in *pex1* mutants (Fig. 3A), increasing PEX5 levels did not ameliorate *pex1* mutant defects, suggesting that these defects are not solely caused by reduced delivery of PEX5 cargo to the peroxisome. The exacerbation of PTS2-processing defects upon PEX5 overexpression in *pex1-2* (Fig. 5C) is also observed in *pex4-1* (Kao and Bartel, 2015), suggesting that PEX5 that is not recycled or degraded lingers in the peroxisomal membrane to the detriment of peroxisome function.

Although PEX1 and PEX6 are related proteins (Fig. 1D) that cooperate to retrotranslocate PEX5 from the membrane (Platta et al., 2005), they also have distinct functions. For example, PEX6, but not PEX1, binds PEX26 to tether the PEX1-PEX6 complex to the peroxisome (Goto et al., 2011). We found that *PEX1*, but not *PEX6*, overexpression in wild-type plants conferred growth defects (Fig. 4G and H), even though PEX1 accumulated at lower levels relative to endogenous PEX1 (Fig. 4D and E) than PEX6 (Fig. 7C). These observations suggest that PEX1 levels cannot exceed a particular level or ratio relative to PEX6. Human PEX1 can form homo-trimers in the cytosol (Tamura et al., 2006) that bind PEX5 (Tamura et al., 2014), suggesting that excessive PEX1 may sequester PEX5 in the cytosol and prevent cargo delivery, leading to the observed defects. Additionally, because *PEX1* was expressed from the constitutive 35S promoter, defects could be due to ectopic expression in tissues that usually do not have high PEX1 levels.

Missense mutations can lead to degradation of the mutant protein. Certain human *pex1* missense mutants can be ameliorated by molecular chaperones that restore PEX1 levels (Zhang et al., 2010) or by lower growth temperatures that decrease degradation (Imamura et al., 1998a; Imamura et al., 1998b; Zhang et al., 2010) and restore PEX1 levels (Tamura et al., 2001; Maxwell et al., 2002). Similarly, *Arabidopsis* PEX1 levels were reduced in *pex1-2* (Fig. 4D, 5B and C, 6C, 7C, and 8B). Lowering the growth temperature ameliorated this reduction, and increasing temperature exacerbated this reduction (Fig. 6C), suggesting that the Leu993Phe mutation destabilizes the pex1-2 protein. Interestingly, PEX6 levels also were reduced in *pex1-2* (Fig. 3B, 4D, 5B and D, 6C, 7C, and 8B), especially at elevated growth temperature (Fig. 6C) suggesting that PEX1 promotes PEX6 stability. Moreover, *PEX6* overexpression restored pex1-2 accumulation (Fig. 7C), suggesting that increasing pex1-2-PEX6 complex formation stabilized the mutant pex1-2 protein. IBA responsiveness and PTS2 processing in *pex1-2* were worsened by growth at high temperature (Fig. 6A and B) and restored by overexpressing *PEX6* (Fig. 7), indicating that the Leu993Phe mutation does not prevent PEX1 function if sufficient pex1-2 protein is present. Similarly, *PEX6* overexpression rescues a *pex1* missense mutation but not a *pex1* null mutation in humans (Geisbrecht et al., 1998), and *PEX6* overexpression rescues a temperature-sensitive *Pichia pastoris* *pex1* mutation (Faber et al., 1998).

Despite the proximity of the *pex1-2* and *pex1-3* mutations (Fig. 1D), the distinct mutant phenotypes indicate different effects on the encoded proteins. *pex1-2* decreased PEX1 levels and
the various *pex1-2* defects all appear to stem from reduced *PEX1* (and *PEX6*) levels (Fig. 6 and 7). In contrast, *PEX1* and *PEX6* protein levels resembled wild-type levels in *PEX1/pex1-3*, suggesting that *pex1-3* might be stable. Indeed, the semidominance of *pex1-3* implies that the *pex1-3* protein is sufficiently stable to incorporate into and poison the PEX1-PEX6 complex, at least in young seedlings.

Unlike the rescue seen when overexpressing *PEX6* in *pex1-2* (Fig. 7), we failed to isolate *pex6-1* plants overexpressing *PEX1*. This result contrasts with findings in human cells, where *PEx1* overexpression can rescue a *pex6* missense mutation (Geisbrecht et al., 1998). However, these findings are probably allele-specific; *PEX6* levels are not low in *pex6-1* (Fig. 3B, 5B, and 6C; Ratzel et al., 2011), suggesting that *pex6-1* is stable and thus not aided by additional *PEX1*. The detrimental effects of *PEx1* overexpression observed in wild type may exacerbate *pex6-1* defects, preventing our isolation of *pex6-1* plants overexpressing *PEX1*.

Our data are consistent with the model of a PEX1-PEX6 heterohexamer that sustains peroxisomal function by retrotranslocating PEX5 for recycling and preventing precocious pexophagy, suggesting that *PEX1* and *PEX6* functions are conserved in *Arabidopsis*. The unique *pex1* alleles described here represent valuable tools to further elucidate both evolutionarily conserved and plant-specific *PEX1* functions in a multicellular organism.

### MATERIALS AND METHODS

#### Plant materials

*Arabidopsis thaliana* wild type and mutants were in the Columbia-0 (Col-0) background. *pex1-2* was isolated in a screen for IBA resistance in dark-grown hypocotyl elongation (Strader et al., 2011) from the progeny of seeds mutagenized by soaking for 16 h in 0.05% (v/v) methyl methanesulfonate. Whole-genome sequencing of three pooled backcrossed lines displaying IBA resistance and PTS2-processing defects revealed *pex1-2* (Supplemental Fig. 1A; Supplemental Data Set 1). A *PEX1/pex1-3* heterozygote was isolated from the progeny of seeds mutagenized by soaking in 0.24% (v/v) ethyl methanesulfonate (EMS) for 16 h in a microscopy-based screen for seedlings displaying cotyledon GFP-PTS1 mislocalization (Rinaldi et al., 2016). Recombination mapping using individuals selected for IBA resistance in hypocotyl elongation that had progeny with GFP-PTS1 mislocalization, 2,4-DB resistance, and PTS2-processing defects suggested a causal heterozygous mutation at the top of chromosome 5 (Fig. 1C). We sequenced genomic DNA of two backcrossed lines displaying segregating cytosolic GFP-PTS1 mislocalization. Among mutations common to both backcrossed lines we found a heterozygous *pex1-3* mutation (Supplemental Fig. 1B; Supplemental Data Set 1). *pex1-2* and *PEX1/pex1-3* were backcrossed at least once prior to phenotypic analysis.
To visualize peroxisomes we used a line expressing GFP-PTS1 driven by the constitutive cauliflower mosaic virus 35S promoter (35S:GFP-PTS1). This line and 35S:PEX5, pex6-1, pex6-1 35S:GFP-PTS1, and pex6-1 35S:PEX5 (Zolman and Bartel, 2004), and atg7-3 GFP-PTS1 (Lai et al., 2011; Farmer et al., 2013) were previously described. We crossed 35S:GFP-PTS1 to pex1-2 and isolated a homozygous line of pex1-2 35S:GFP-PTS1 by following the construct in the progeny using fluorescence and PCR-based genotyping (Table 1). We crossed atg7-3 GFP-PTS1 to PEX1/pex1-3 to obtain PEX1/pex1-3 atg7-3/atg7-3 GFP-PTS1.

To generate overexpressing lines, a PEX1 cDNA in pCR8/GW/TOPO (Goto et al., 2011) and a PEX6 cDNA in pENTR223 (stock G21748) from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University were cloned into pEarleyGate destination vectors pEG100 and pEG201 (Earley et al., 2006) from the ABRC using clonase (Invitrogen). The resultant 35S:PEX1, 35S:HA-PEX1, and 35S:HA-PEX6 plasmids were used to transform Agrobacterium tumefaciens GV3101 (pMP90) (Konecz and Schell, 1986) by electroporation. Transformed Agrobacterium strains were used to transform pex1-2 plants using the floral dip method (Clough and Bent, 1998) to obtain pex1-2 35S:PEX1, pex1-2 35S:HA-PEX1, and pex1-2 35S:HA-PEX6. We crossed pex1-2 35S:HA-PEX1 and pex1-2 35S:PEX1 to wild type and obtained wild-type 35S:HA-PEX1 and wild-type 35S:PEX1, respectively. We also crossed pex1-2 35S:PEX1 to PEX1/pex1-3 and obtained pex1-3 35S:PEX1 and crossed wild-type 35S:PEX5 to pex1-2 and to PEX1/pex1-3 to obtain pex1-2 35S:PEX5 and PEX1/pex1-3 35S:PEX5, respectively. Homozygous lines were selected from the progeny of crosses using glufosinate ammonium (Basta) resistance and PCR-based genotyping (Table 1).

Growth conditions and phenotypic assays

Seeds were surface-sterilized and stratified at 4 °C for 1 d before sowing on plates containing plant nutrient medium (Haughn and Somerville, 1986) solidified with 0.6% (w/v) agar and supplemented with 0.5% (w/v) sucrose. Plates were sealed with gas-permeable tape and incubated at 22 °C under continuous white fluorescent light unless otherwise noted. Light was filtered through yellow long-pass filters when treating with hormones to slow indolic compound breakdown (Stasinopoulos and Hangarter, 1990). The same volume of ethanol was added to control media when using ethanol-dissolved hormones (IBA, 2,4-DB, and NAA). Hypocotyl lengths were measured on seedlings grown on plates that were incubated under light for 1 d and then wrapped in aluminum foil and incubated for 4 d. Lateral roots that protruded from the epidermis were counted on seedlings that were grown on medium without hormone for 4 d and then transferred to media with or without hormone and grown for an additional 4 d. For assays that measured single individuals, progeny of PEX1/pex1-3 heterozygous plants were measured and then individually genotyped to identify heterozygotes. Plants were transferred to soil (Sun...
Gro Metro-Mix 366) after 14-16 d on plates and grown at 22 °C under continuous white
fluorescent light.

**DNA analysis**

PCR was performed on DNA that was prepared as described (Celenza et al., 1995). For
recombination mapping of pex1-3, progeny from an outcross to the *Arabidopsis thaliana*
Landsberg erecta (Ler) accession were genotyped using PCR-based markers that exploit
polymorphisms between Col-0 and Ler DNA (Table 2).

Genomic DNA for whole-genome sequencing was prepared as previously described (Thole
et al., 2014) and sent to the Genome Technology Access Center at Washington University in St.
Louis for sequencing with Illumina HiSeq 2000 sequencers. The *Arabidopsis* Col-0 genome
from The *Arabidopsis* Information Resource (TAIR build 10) was used with Novoalign
(Novocraft; http://novocraft.com) to align the sequences and SAMtools (Li et al., 2009) and
snpEFF (Cingolani et al., 2012) were used to identify mutations. Mutations that were previously
found in our lab Col-0 line (Farmer et al., 2013) were disregarded. Sequencing coverage for
*pex1-2* was ≥10X for 71% of the genome and ≥5X for 85% of the genome; coverage for *pex1-3*
was ≥10X for 86-88% of the genome and ≥5X for 99% of the genome.

**Microscopy**

Seedpods, seeds, and seedlings were imaged with a Leica ZM10 F dissecting microscope
equipped with a Leica DFC295 camera.

For visualizing GFP-PTS1 localization, cotyledons of light-grown seedlings were mounted in
water and imaged with a Carl Zeiss LSM 710 laser scanning confocal microscope using a 63X
oil immersion objective, a Meta detector, and the ZEN 2010 version 6.0.0.485 software. A 488-
nm argon laser was used for excitation, and GFP fluorescence emission was collected between
493-526 nm through a 24-µm pinhole, corresponding to a 1-µm optical slice. Each image is an
average of four exposures. Fluorescence was imaged at two different planes in epidermal cells:
midway through the cell (mid-cell), where cytosolic fluorescence outlines the cell, and
immediately below the plasma membrane (sub-cortical), where cytosolic fluorescence is
distributed across the plane of acquisition.

**Immunoblotting**

Frozen plant tissue was ground in two volumes of 2X NuPAGE sample buffer (Invitrogen,
Carlsbad, CA) and centrifuged at 16,100 g for 5 min. Dithiothreitol was added to the
supematants to a final concentration of 50 mM and samples were incubated at 100 °C for 5 min.
15 µL of each sample was loaded on NUPAGE or BOLT 10 % Bis-Tris gels (Invitrogen)
alongside Cruz molecular mass markers (sc-2035; Santa Cruz Biotechnology, Santa Cruz, CA)
and prestained protein markers (P7708; New England Biolabs, Beverly, MA) and subjected to
electrophoresis using 1X MOPS running buffer [50 mM 3-(N-morpholino)-propanesulfonic acid,
50mMTris base, 0.1% sodium dodecyl sulfate, 1 mM EDTA]. Gels were transferred to Hybond
nitrocellulose membranes (Amersham Pharmacia Biotech) using NuPAGE transfer buffer
(Invitrogen). Membranes were blocked in 8% non-fat dry milk in TBST (20 mM Tris pH 7.5,
150 mM NaCl, and 0.1% Tween 20) for 1 h at 4 °C with rocking. After blocking, membranes
were incubated overnight at 4 °C with primary antibodies in blocking solution with 0.1% sodium
azide at the indicated dilutions: rabbit antibodies against PMDH2 ([1:1,500; Pracharoenwattana et
al., 2007], the PED1 isoform of thiolase (1:10,000; Lingard et al., 2009), PEX1 (1:200; raised to
the first 400 aa of Arabidopsis PEX1 and affinity purified by Proteintech Group), PEX5 (1:100;
Zolman and Bartel, 2004), and PEX6 (1:1,000; Ratzel et al., 2011); mouse antibodies against
HSC70 (1:50,000; Stressgen SPA-817) and GFP (1:100; Santa Cruz Biotechnology sc-9996); or
a rat antibody against HA (1:100, Roche 3F10). Membranes were washed with TSBT and
incubated for 4 h at 4 °C with secondary horseradish peroxidase-linked goat antibodies against
rabbit, mouse, or rat (1:5,000; Santa Cruz Biotechnology sc-2030, sc-2031, and sc-2032,
respectively) diluted in blocking solution. Membranes were washed and incubated with
WesternBright ECL (Advansta) to visualize horseradish peroxidase activity by exposing
membranes to autoradiography film. Films were imaged using a scanner. Various primary
antibodies were used sequentially to probe membranes without stripping.

Fractionation

Cell fractionation was performed as previously described (Ratzel et al., 2011).

Approximately 500 mg of tissue was chopped with scissors in 1 mL ice-cold fractionation buffer
[150 mM Tris pH 7.6, 100 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM
N-ethylmaleimide, 1 mM phenylmethyl sulfonyl fluoride, 1X protease inhibitor cocktail (Sigma
P9599)], processed with a Dounce homogenizer (20 strokes) and filtered through Miracloth
(Millipore). Samples were centrifuged for 10 min at 4,300 g and a third of the supernatant
(around 150 µL) was used as the homogenate fraction (H). The rest of the volume (around 300
µL) was centrifuged for 20 min at 13,400 g and most of the new supernatant (around 240 µL)
was collected as the supernatant fraction (S). The pellet was re-suspended in twice the
homogenate volume (around 300 µL) of fractionation buffer, centrifuged for 20 min at 13,400 g
and most of the new supernatant (around 240 µL) was collected as the wash fraction (W). The
remaining supernatant was discarded and the pellet was re-suspended in twice the homogenate
volume (around 300 µL) of fractionation buffer to be used as the pellet fraction (P). Volumes
were adjusted according to the volume obtained in the first centrifugation to maintain a ratio of
H:S:P:W of 5:8:10:8. Each fraction was mixed with a third volume of NuPAGE 4X loading buffer (Invitrogen) and 40 µL were used for immunoblotting as described above.

Statistical analysis

When more than two samples were compared, the SPSS Statistics software (version 22.0.0.1) was used to analyze differences among samples in a particular treatment group using one-way ANOVA followed by Duncan’s test. Mean values that were significantly different from each other (p < 0.001) are marked with a different letter above the bar.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: *Arabidopsis* PEX1 (At5g08470), *Arabidopsis* PEX6 (At1g03000), human PEX1 (NP_000457.1), human PEX6 (NP_000278.3), and mouse p97 (NP_033529.3).

Supplemental Material

**Supplemental Fig. 1.** Mutations identified through whole-genome sequencing of *pex1* mutant DNA.

**Supplemental Fig. 2.** PEX5 is distributed like wild type between cytosolic and organellar fractions in *pex1* mutants.

**Supplemental Data Set 1.** Mutations identified through whole-genome sequencing.

**Supplemental Fig. 1.** Mutations identified through whole-genome sequencing of *pex1* mutant DNA.

**Supplemental Fig. 2.** PEX5 is distributed like wild type between cytosolic and organellar fractions in *pex1* mutants.

**Supplemental Data Set 1.** Mutations identified through whole-genome sequencing. Changes in splice sites or nonsynonymous amino acid changes in coding regions found indicating chromosome number (Chr), reference (R) and changed (C) nucleotide, and zygosity (Zyg). Only EMS-consistent mutations are shown for *pex1*-3. Descriptions obtained from TAIR. Homozygous mutations are in red text; presumptive causal mutations are highlighted in yellow.

ACKNOWLEDGEMENTS

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use as an *Arabidopsis* mutagen. We are grateful to Yun-Ting Kao, Roxanna Llinas, Zachary Wright, and Pierce Young for critical comments on the manuscript.
Table 1: PCR-based markers used to genotype identified mutants, reference lines, and transgenes

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*Underlined base indicates difference from the original sequence to insert an Rsal site in the wild-type amplicon.

Table 2: PCR-based markers used for recombination mapping of pex1-3

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<td>MWD9-2 (CTGGGCTCTCTACCTGATAC)</td>
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<td></td>
<td>GA3-2 (CCGAACGCTCTTATCCCATGTTGC)</td>
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biogenesis and plays critical roles during pollen maturation and germination in planta. Plant Cell 26, 619-635.


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