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Matrix proteins are inefficiently imported into *Arabidopsis* peroxisomes lacking the receptor-docking peroxin PEX14

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Abstract

Mutations in peroxisome biogenesis proteins (peroxins) can lead to developmental deficiencies in various eukaryotes. PEX14 and PEX13 are peroxins involved in docking cargo-receptor complexes at the peroxisomal membrane, thus aiding in the transport of the cargo into the peroxisomal matrix. Genetic screens have revealed numerous *Arabidopsis thaliana* peroxins acting in peroxisomal matrix protein import; the viable alleles isolated through these screens are generally partial loss-of-function alleles, whereas null mutations that disrupt delivery of matrix proteins to peroxisomes can confer embryonic lethality. In this study, we used forward and reverse genetics in *Arabidopsis* to isolate four *pex14* alleles. We found that all four alleles conferred reduced *PEX14* mRNA levels and displayed physiological and molecular defects suggesting reduced but not abolished peroxisomal matrix protein import. The least severe *pex14* allele, *pex14-3*, accumulated low levels of a C-terminally truncated PEX14 product that retained partial function. Surprisingly, even the severe *pex14-2* allele, which lacked detectable *PEX14* mRNA and

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PEX14 protein, was viable, fertile, and displayed residual peroxisome matrix protein import. As *pex14* plants matured, import improved. Together, our data indicate that PEX14 facilitates, but is not essential for peroxisomal matrix protein import in plants.

Keywords

Peroxin; Peroxisome import; Organelle targeting; *Arabidopsis* molecular genetics

Introduction

Eukaryotic organelles employ distinct mechanisms to ensure targeting of proteins synthesized in the cytosol to appropriate subcellular destinations. Proteins housed in peroxisomes are imported through the action of peroxin (PEX) proteins. The PEX5 and PEX7 receptors recognize cytosolic cargo proteins with type 1 or type 2 peroxisomal targeting signals (PTS), respectively. PTS-bearing proteins are translocated into the peroxisome upon receptor binding to a docking complex, which includes the interacting membrane peroxins PEX13 and PEX14 in mammals, plants, and yeasts (reviewed in Lanyon-Hogg et al. 2010). Recent in vitro evidence suggests that yeast PEX5, when recruited to the peroxisomal membrane by PEX14, forms the pore through which PTS1 proteins enter the peroxisome (Meinecke et al. 2010). However, much remains unknown about how proteins enter peroxisomes and the extent to which peroxisomal import mechanisms have diverged during eukaryotic evolution.

In yeast, PEX5-dependent PTS1 import and PEX7-dependent PTS2 import converge at the PEX14-PEX13 docking complex; mutants defective in either docking peroxin exhibit both PTS1 and PTS2 import defects (Elgersma et al. 1996; Albertini et al. 1997). In *Saccharomyces cerevisiae*, PEX14 and PEX13 each can bind directly to PEX5 and PEX7 and to each other (Elgersma et al. 1996; Albertini et al. 1997; Stein et al. 2002; Niederhoff et al. 2005). The in vitro binding of *Pichia pastoris* PEX5 to PEX14 is enhanced by PTS1 cargo, whereas PEX5-PEX13 binding is diminished by PTS1 cargo (Urquhart et al. 2000), suggesting that PEX14 is the initial docking site for PEX5-PTS1 cargo complexes and that PEX13 acts after cargo release. Conversely, recent studies in *S. cerevisiae* suggest that the PEX7-PTS2 import pathway uses PEX13 as the initial docking site followed by interaction with PEX14 (Grunau et al. 2009).

In humans, *PEX14* lesions underlie a subset of peroxisome biogenesis disorders (Shimozawa et al. 2004; Huybrechts et al. 2008). As in yeast, mammalian PEX14 interacts with PEX5, PEX7, and PEX13 (reviewed in Azevedo and Schliebs 2006). In contrast to yeast, where PEX5 and PEX7 can independently interact with the docking peroxins and deliver PTS1 and PTS2 cargo, respectively, mammalian PTS2 cargo proteins require a long version of PEX5 (PEX5L) to enter peroxisomes (Braverman et al. 1998; Matsumura et al. 2000; Dodt et al. 2001). PEX5L facilitates the in vitro binding of PEX7-PTS2 cargo complexes to PEX14 (Otera et al. 2000). Although mammalian PEX14 can interact with PEX7 in vitro (Shimizu et al. 1999), immunoprecipitation assays demonstrate that PEX7 primarily associates with PEX13, whereas PEX5L primarily associates with PEX14 (Miyata et al. 2009).

Similar to mammals, *Arabidopsis* PTS2 protein import depends not only on PEX7, but also on PEX5, the PTS1 receptor (Hayashi et al. 2005; Woodward and Bartel 2005), and *Arabidopsis* PEX7 binds PEX5 in yeast two-hybrid assays (Nito et al. 2002; Ramón and Bartel 2010). The *Arabidopsis pex5-1* missense allele (Zolman et al. 2000), which alters a conserved amino acid residue that in mammalian PEX5L is essential for PEX7 binding and

PTS2 import (Matsumura et al. 2000), impairs PTS2 but not PTS1 import (Woodward and Bartel 2005). In *Arabidopsis*, PEX5 levels and PTS1 import depend on PEX7 function in certain growth conditions (Ramón and Bartel 2010), suggesting that PEX5 is stabilized in the presence of PEX7. As in other eukaryotes (Schliebs et al. 1999; Otera et al. 2002), the N-terminal region of *Arabidopsis* PEX14 binds PEX5 (Nito et al. 2002), however, direct PEX14-PEX7 interactions have not been reported in plants, and *Arabidopsis* PEX14 fails to bind PEX7 in yeast two-hybrid assays (Nito et al. 2002). PEX14-blocking antibodies prevent in vitro binding of both PTS1- and PTS2-cargo to sunflower peroxisomes (Lopez-Huertas et al. 1999), and *Arabidopsis* mutants defective in either PEX14 (Hayashi et al. 2000) or PEX13 (Mano et al. 2006) have deficiencies in both PTS1 and PTS2 import, implying that PEX5 and PEX7 require both docking peroxins for efficient cargo translocation into the peroxisome. Moreover, the N-terminal portion of *Arabidopsis* PEX13 interacts with PEX7, but not PEX5, in a yeast two-hybrid assay (Mano et al. 2006). These interaction studies suggest that plants, like mammals (Miyata et al. 2009), maintain distinct PEX14-PEX5 and PEX13-PEX7 relationships despite the interdependence of PTS1 and PTS2 pathways in plants (Ramón and Bartel 2010).

Notwithstanding the demonstrated importance of PEX14 and PEX13 in both PTS1 and PTS2 import, *Arabidopsis* mutants defective in either docking complex peroxin have disparate phenotypes. A *PEX13* null allele confers lethal gametophytic defects and completely blocks peroxisomal import of a PTS1-tagged reporter protein in pollen (Boisson-Dernier et al. 2008). In contrast, the single characterized *pex14* mutant, *peroxisome defective2* (*ped2*), displays more mild defects despite harboring a nonsense mutation midway through the *PEX14* coding sequence (Hayashi et al. 2000). Although *ped2* peroxisomes are shrunken and *ped2* plants are small and display photorespiration defects, light-grown *ped2* root cells display only partial GFP-PTS1 import defects, and dark-grown *ped2* seedlings exhibit only partial PTS2 processing defects (Hayashi et al. 2000). Moreover, PTS2 processing is restored as *ped2* seedlings mature (Hayashi et al. 2000). These partial and transient matrix protein import defects suggest either that the *ped2* allele does not completely eliminate PEX14 function or that PEX14 is not absolutely required for matrix protein import. Moreover, the *ped2* accession (Landsberg) precludes direct phenotypic comparison to other *Arabidopsis pex* mutants, which have been isolated in the Columbia accession. Here we describe the physiological, molecular, and cell biological defects in an allelic series of *Arabidopsis pex14* mutants in the Columbia accession. Our analysis of these mutants, including an apparent null allele, suggests that PEX14 facilitates, but is not absolutely required for matrix protein import into *Arabidopsis* peroxisomes.

Results

An allelic series of *pex14* mutants

Plant peroxisomes house processes essential for growth and development, including fatty acid β-oxidation, which fuels early seedling growth (Baker et al. 2006), and the related process of indole-3-butyric acid (IBA) conversion to the active auxin indole-3-acetic acid (IAA) (Zolman et al. 2000; Strader et al. 2010; Strader and Bartel 2011). As a result, screens for resistance to IBA or synthetic auxin precursors have facilitated the isolation and characterization of *Arabidopsis* mutants with defective peroxisomes (Hayashi et al. 1998; Zolman et al. 2000). We isolated four *Arabidopsis pex14* alleles, two in forward genetic screens for reduced peroxisomal function and two from a T-DNA insertion collection. *pex14-1* was isolated as an IBA-response mutant from the progeny of plants transformed with a T-DNA based cDNA library (LeClere and Bartel 2001). Because the mutant lesion was not linked to the T-DNA, we used recombination mapping to localize the causal mutation near the bottom of chromosome 5 (Fig. 1a). The *PEX14* (*At5g62810*) gene is in this interval, and because the *pex14* mutant *peroxisome defective2* (*ped2*, Hayashi et al.

2000) has a similar phenotype (see below), we sequenced *PEX14* from the mutant genomic DNA. We found a 19-bp deletion beginning at position 1722 (where 1 is the A of the initiator ATG) that resulted in one out-of-frame amino acid followed by an early stop codon (Fig. 1b).

We isolated *pex14-4* in a screen of ethyl methanesulfonate-mutagenized pools for seedlings that required sucrose for early seedling development and that failed to efficiently produce lateral roots in response to IBA, two hallmarks of peroxisome-defective mutants (Zolman et al. 2000). This mutant was mapped to a similar interval as *pex14-1* near the bottom of chromosome 5 (Fig. 1a). Sequencing *PEX14* from mutant DNA revealed a G-to-A mutation at position 2,220 that altered the 5′ splice site of the eighth intron. Skipping this intron would result in 19 out-of-frame amino acids followed by an early stop codon (Fig. 1b).

To expand our allelic series, we obtained two additional *pex14* alleles from the Salk Institute collection of sequence-tagged lines (Alonso et al. 2003). *pex14-2* has a T-DNA insertion 41-bp downstream of the *PEX14* start codon, and *pex14-3* has a T-DNA insertion 267-bp upstream of the *PEX14* stop codon (Fig. 1b).

Arabidopsis PEX14 is a 507-amino acid protein that migrates atm ~75 kDa in SDS–PAGE (Hayashi et al. 2000) and resembles PEX14 from other organisms, with an N-terminal PEX5-binding domain, a hydrophobic region that may anchor the protein in the peroxisomal membrane, and a predicted coiled-coil region (reviewed in Azevedo and Schliebs 2006). The *pex14* alleles are distributed throughout the coding sequence, with the *pex14-2* allele disrupted upstream of the identified domains, *pex14-1* and *pex14-4* after the hydrophobic region near the middle of the protein, and *pex14-3* near the C-terminus (Fig. 1c). We used RNA gel-blot analysis to examine seedling *PEX14* mRNA levels and did not detect full-length *PEX14* transcript in either T-DNA allele (*pex14-2* and *pex14-3*; Fig. 1d). Moreover, *pex14-1* and *pex14-4* displayed reduced *PEX14* transcript levels compared to wild type (Fig. 1d), consistent with the possibility that the early stop codons in these alleles (Fig. 1b) led to nonsense-mediated mRNA decay (Stalder and Muhlemann 2008). To determine the consequences of the *pex14* mutations on PEX14 protein accumulation, we examined extracts from *pex14* plants using an antibody generated against the N-terminal 243 amino acids of *Arabidopsis* PEX14 (Lingard and Bartel 2009), which we expected would detect any accumulated pex14-1 protein, because the *pex14-1* premature stop codon occurs one amino acid residue following Tyr241 (Fig. 1b), as well as pex14-3, and pex14-4 proteins, which would be larger than pex14-1 (Fig. 1c). As expected, none of the *pex14* mutants accumulated detectable full-length PEX14 (Fig. 1e). However, we did detect reduced levels of truncated versions of PEX14 in the *pex14-1* and *pex14-3* mutants (Fig. 1e) migrating at sizes consistent with the location of the mutant lesions (Fig. 1c). Our observation that the truncated PEX14 products did not accumulate to wild-type PEX14 levels is consistent with the reduced *pex14* transcript levels in these mutants (Fig. 1d). Our finding that detectable *PEX14* mRNA or PEX14 protein did not accumulate in the *pex14-2* T-DNA allele (Fig. 1d, e), in combination with the position of the insertion (Fig. 1b), strongly suggests that *pex14-2* is a null allele.

We also used immunoblotting to determine whether reduced PEX14 levels impacted levels of other peroxins. Certain *pex* mutants, including *pex6-1* (Zolman and Bartel 2004) and *pex7-2* (Ramón and Bartel 2010) accumulate less PEX5 protein than wild type. We detected similar levels of PEX5, PEX6, PEX7, and PEX13 in 8-day-old *pex14* and wild-type seedlings (Fig. 1e), suggesting that these peroxins do not require PEX14 for stability. Conversely, PEX14 accumulation is similar in wild type and in *pex4*, *pex5*, *pex6*, and *pex7* mutants (Ratzel et al. 2011), suggesting that PEX14 does not depend on the corresponding peroxins for stability.

Reduced IBA responsiveness in *pex14* mutants

We assayed IBA responsiveness to compare the physiological severity of the *pex14* alleles to two previously characterized PTS1 receptor mutants, the *pex5-1* missense allele (Zolman et al. 2000; Woodward and Bartel 2005) and the *pex5-10* T-DNA insertion allele (Zolman et al. 2005; Khan and Zolman 2010; Ramón and Bartel 2010). Genetic and biochemical data suggest that IBA is β-oxidized to IAA in peroxisomes and that this conversion is necessary for response to exogenous IBA (Zolman et al. 2000, 2007, 2008; Strader et al. 2010, 2011). Consequently, IBA responses, such as the inhibition of primary root elongation and the promotion of lateral root formation, can be used to assess peroxisome function (Zolman et al. 2000). We found that the *pex14* mutants were slightly less IBA resistant than *pex5-1* and *pex5-10* in root elongation assays. Like *pex5-1* and *pex5-10*, the four *pex14* mutants displayed complete resistance to the inhibitory effects of 10 μM IBA on root elongation. However, *pex14* mutants were only partially resistant to 20 μM IBA, whereas both *pex5* mutants still showed substantial resistance at this concentration (Fig. 2a). All of the mutants responded to 40 μM IBA (Fig. 2a).

pex14 mutants also displayed lateral root production defects in response to IBA. We counted lateral roots of 8-day-old seedlings grown for 4 days on medium without hormone followed by 4 days on medium supplemented with either IBA or the synthetic auxin 1-naphthaleneacetic acid (NAA), which does not require peroxisomal chain shortening for activity. Wild-type seedlings had few lateral roots without auxin treatment and increased numbers of lateral roots following either IBA or NAA treatment (Fig. 2b). Like wild-type seedlings, the *pex14* mutants responded to NAA by forming lateral roots (Fig. 2b). In response to IBA, however, the *pex14* mutants displayed dramatically fewer lateral roots than wild type (Fig. 2b). This phenotype is consistent with the lateral root deficiency of other *pex* mutants (Zolman et al. 2000, 2005; Zolman and Bartel 2004; Woodward and Bartel 2005; Ramón and Bartel 2010), including *pex5-1* and *pex5-10* (Fig. 2b).

pex14 growth defects

Peroxisomes are the sole organelles housing fatty acid β-oxidation in plants (reviewed in Graham 2008), and mutants defective in β-oxidation of seed storage fatty acids typically display growth defects following germination that can be at least partially restored by exogenous sucrose (Hayashi et al. 1998; Zolman et al. 2000), presumably because this fixed carbon source replaces the energy normally provided by the acetyl-CoA freed during β-oxidation of stored fatty acids. To indirectly assess the ability of *pex14* seedlings to metabolize endogenous oil reserves, we monitored seedling growth on media with and without supplemented sucrose. Whereas dark-grown *pex14* hypocotyls were of nearly wild-type lengths when seedlings were germinated and grown with 0.5% sucrose, we found dramatic *pex14* hypocotyl elongation defects in the absence of sucrose (Fig. 3a). The *pex14-2*, *pex14-1*, and *pex14-4* mutants were more severely sucrose dependent than *pex14-3* (Fig. 3a).

We also observed sucrose dependence in light-grown *pex14* seedlings. The slight root elongation defect of *pex14* mutants grown on sucrose in the light was exacerbated in the absence of sucrose (Fig. 3b). The sucrose dependence of light-grown *pex14* seedlings was observed not only in root elongation (Figs. 3b, 4a, b), but also in leaf expansion (Fig. 4a, b). Like sucrose dependence in the dark, the root and shoot expansion defects were least severe in the *pex14-3* mutant (Fig. 3b).

Even when supplied with sucrose, *pex14* mutants were smaller than wild type and *pex5-1* (Fig. 4b). Although this defect persisted after plants were transferred to soil (Fig. 4c–e), all of the *pex14* mutants were fertile and produced viable seeds. Initially, the *pex14* mutants

were less delayed than *pex5-10* when grown in soil (Fig. 4d), but at maturity, three of the *pex14* mutants (*pex14-2*, *pex14-1*, and *pex14-4*) were smaller than *pex5-10*, with small rosette leaves and reduced stature (Fig. 4d, e). As with other phenotypes, the *pex14-3* allele conferred less severe growth defects than the other *pex14* lesions (Fig. 4).

In summary, our analyses of *pex14* physiological phenotypes revealed growth defects in the presence and absence of sucrose that were generally intermediate between the moderate defects of *pex5-1* and the severe defects of *pex5-10*, both in the light and the dark (Figs. 3, 4a, b). Among the *pex14* mutants, several defects appeared less severe in *pex14-3* than in *pex14-2*, *pex14-1*, or *pex14-4* (Figs. 3, 4), suggesting that the pex14-3 protein (Fig. 1e) retained partial PEX14 function and that the other *pex14* lesions conferred more complete disruptions.

pex14 defects in PTS2 protein processing

We used immunoblot analyses to determine whether the physiological defects displayed by the *pex14* mutants were accompanied by defects in matrix protein import. Because the PTS2-containing signal is removed from PTS2 cargo proteins by the DEG15 peroxisomal protease after cargo enters the peroxisome, and because the DEG15 protease itself is a PTS1 protein (Helm et al. 2007; Schuhmann et al. 2008), monitoring PTS2 removal indirectly assesses per-oxisomal import of PTS1 and/or PTS2 proteins. We analyzed PTS2 processing of 3-ketoacyl-CoA thiolase (thiolase) and peroxisomal malate dehydrogenase (PMDH) in seedlings and leaves of mature plants. As previously reported (Woodward and Bartel 2005; Zolman et al. 2005; Ramón and Bartel 2010), both proteins were fully processed in wild type and displayed marked processing defects in both *pex5* mutants (Fig. 5a). All four *pex14* mutants displayed PTS2 processing defects; these defects appeared less severe in the *pex14-3* mutant. Interestingly, the *pex14* PTS2 processing defects were less severe than either *pex5-1* or *pex5-10* in both 3- and 5-day-old seedlings (Fig. 5a). In particular, unprocessed thiolase was a minor fraction of total thiolase in 5-day-old *pex14* seedlings, whereas less than half of the thiolase was processed in either *pex5-1* or *pex5-10* at 5 days (Fig. 5a). Like thiolase, more than half of the PMDH was processed in 3-day-old *pex14* seedlings, whereas the bulk of PMDH was unprocessed in 3-day-old *pex5-1* and *pex5-10* seedlings (Fig. 5a). In contrast to thiolase processing defects, PMDH processing defects appeared more severe in 5-day-old *pex14* seedlings than in 3-day-old seedlings, perhaps because PMDH levels are increasing from 3 to 8 days after sowing while thiolase levels are declining (Lingard et al. 2009). Like thiolase, PMDH processing defects were not as severe in any of the *pex14* mutants as the defects detected in either *pex5* mutant.

The recovery of thiolase PTS2 processing ability as *pex14* seedlings matured from 3 to 5 days old (Fig. 5a) was consistent with the previous characterization of the *ped2* allele of *pex14*, which also displays only transient thiolase processing defects (Hayashi et al. 2000). In contrast to thiolase, however, the partial PMDH processing defects were maintained as *pex14* seedlings matured from 3 to 9 days old (Fig. 5a, b). In mature plants, however, PMDH processing defects were no longer apparent in the *pex14-3* mutant and were reduced in the other *pex14* mutants compared to *pex5-1*, which still processed less than half of the PMDH in 35-day-old plants (Fig. 5c). Thus the recovery of PTS2 processing efficiency seems not to be a specific feature of thiolase, but may reflect a general improvement as *pex14* mutants mature in either PTS1 import (including import of the DEG15 PTS2-processing protease), PTS2 import, or both.

pex14 defects in peroxisomal matrix protein import

To examine the effects of *pex14* mutations on PTS1 import and to compare the PTS1 import defects of *pex14-2* and *pex14-1*, we used confocal fluorescence microscopy to examine lines

carrying a reporter driven by the isocitrate lyase (*ICL*) promoter in which green fluorescent protein (GFP) is fused to the N-terminus of the PTS1 protein ICL (Lingard et al. 2009). As expected, this reporter was distributed in a punctate pattern in cotyledon cells from 3- and 9-day-old wild-type seedlings (Fig. 6a), consistent with a fully peroxisomal localization. In contrast, GFP-ICL fluorescence appeared largely cytosolic in *pex14-1* seedlings, with some punctate foci barely discernable in 9-day-old seedlings (Fig. 6a). In *pex14-2* seedlings, GFP-ICL fluorescence appeared fully cytosolic at both 3 and 9 days (Fig. 6a). The more complete mislocalization of GFP-ICL in *pex14-2* is consistent with the possibility that the *pex14-2* lesion confers a more severe disruption of PEX14 function and PTS1 import than does the *pex14-1* lesion.

ICL functions in the glyoxylate cycle (Eastmond et al. 2000) and is normally degraded a few days after germination (Lingard et al. 2009). We could still detect ICL and GFP-ICL in *pex14* mutants after these proteins had been mostly degraded in wild type (Fig. 6a, e). This stabilization is consistent with our previous finding that ICL must be imported into the peroxisome to undergo efficient degradation (Lingard et al. 2009).

To compare the effects of a *pex14* mutation on PTS1 and PTS2 matrix protein import, we crossed *pex14-1* to transgenic lines containing PTS1- or PTS2-targeted GFP derivatives driven by the cauliflower mosaic virus *35S* promoter. In 3- and 9-day-old wild-type cotyledon cells, both GFP-PTS1 (Zolman and Bartel 2004) and PTS2-GFP (Woodward and Bartel 2005) appeared in punctate structures indicative of peroxisomes (Fig. 6b, c). Unlike in wild type, both GFP-PTS1 and PTS2-GFP appeared partially cytosolic in 3-day-old *pex14-1* seedlings (Fig. 6b, c). These data confirmed that both PTS1 and PTS2 import is partially defective in *pex14-1*.

Because we saw a recovery of thiolase PTS2 processing as *pex14* seedlings aged (Fig. 5a), we also analyzed import of GFP-PTS1 and PTS2-GFP in older mutant plants. Cotyledon epidermal cells of 9-day-old *pex14-1* plants expressing GFP-PTS1 exhibited both punctate and cytosolic fluorescence, similar to that seen in 3-day-old seedlings (Fig. 6b). In contrast, epidermal cells from 9-day-old *pex14-1* plants expressing PTS2-GFP exhibited primarily punctate fluorescence, with some cytosolic fluorescence persisting only in stomatal cells. Analysis of PTS2 processing in *pex14* transgenic seedlings by immunoblotting confirmed that the PTS2-GFP processing defect became less severe as seedlings aged (Fig. 6d), similar to endogenous thiolase (Figs. 5, 6f). These results suggested that PEX14 might be more important for PTS1 import than for PTS2 import as seedlings mature.

PTS2 processing requires both import of the PTS2 protein into the peroxisome and import of the DEG15 PTS2-processing enzyme, which is a PTS1-containing protein (Helm et al. 2007; Schuhmann et al. 2008). To examine possible causes of *pex14* PTS2 processing defects, we fractionated extracts from 3- and 10-day-old seedlings into cytosolic and organellar fractions (Ratzel et al. 2011). As expected, we found the mitochondrial ATPase in the organellar fraction and most of the HSC70 in the cytosolic fraction of both wild type and *pex14-2* (Fig. 7). When we examined the PTS2 protein PMDH, we found unprocessed PMDH in both the cytosolic and organellar fractions in both 3- and 10-day-old *pex14-2* seedlings (Fig. 7), consistent with the PTS2-GFP import defect in *pex14-1* (Fig. 6c). In the cytosolic fractions, which would include both cytosolic proteins and proteins from organelles that ruptured during fractionation, we observed that more than half of the PMDH was unprocessed in *pex14-2*, whereas less than half of the PMDH was unprocessed in the *pex14-2* organellar fractions. We found that the PTS1 protein malate synthase (MLS), a glyoxylate cycle enzyme that only is present in young seedlings (Lingard et al. 2009), appeared more cytosolic in 3-day-old *pex14-2* seedlings than in wild type (Fig. 7). The reduced organellar association of MLS is consistent with the reduced punctate fluorescence of PTS1-tagged

GFP derivatives (Fig. 6a, b) and suggests that PTS1 import defects may contribute to the PTS2 processing defects of *pex14* mutants. Interestingly, however, substantial MLS remained associated with the *pex14-2* organellar pellet, suggesting that PTS1 import, like PTS2 import, was not fully blocked even in the *pex14-2* presumptive null allele.

To determine if the loss of PEX14 was accompanied by altered receptor localization, we examined PEX5 and PEX7 distribution in the fractions. PEX7 was similarly distributed between the cytosolic and organellar fractions in wild type and *pex14-2* at both 3 and 10 days, and PEX5 was similarly distributed between the cytosolic and organellar fractions at 3 days (Fig. 7). However, the PEX5 shift to the organellar fraction observed in 10-day-old wild-type seedlings did not appear as complete in 10-day-old *pex14-2* seedlings (Fig. 7), suggesting that PEX5 may be targeted to the peroxisome less efficiently in the absence of PEX14.

We also examined the distribution of the docking peroxin PEX13 in *pex14-2* seedling extracts. As expected, we found PEX13 in the organellar pellet in wild-type seedlings (Ratzel et al. 2011) and in 10-day-old *pex14-2* seedlings (Fig. 7). However, we did not detect PEX13 in 3-day-old *pex14-2* seedlings, even in the concentrated organellar fraction, and PEX13 appeared to be less abundant in 10-day-old *pex14-2* seedlings than in wild type (Fig. 7), suggesting that PEX14 may stabilize PEX13 in the peroxisome membrane. Alternatively, because PEX13 accumulates as seedlings mature (Fig. 7), the delayed appearance of PEX13 in the *pex14* mutant may reflect the slow growth of *pex14* seedlings.

Discussion

Most peroxins assist in importing proteins from the cytosol to the peroxisomal matrix, either directly, as cargo-binding receptors (PEX5, PEX7) or docking receptor-cargo complexes at the peroxisome membrane (PEX13, PEX14), or indirectly, by promoting the recycling of receptors back to the cytosol for further import rounds (e.g., PEX1, PEX2, PEX4, PEX6, PEX10, PEX12, PEX22, PEX26). Over the past decade, *Arabidopsis* mutants have been reported that are defective in nearly all of the conserved peroxins implicated in matrix protein import and receptor recycling, including PEX2 (Hu et al. 2002), PEX4 (Zolman et al. 2005), PEX5 (Zolman et al. 2000; Khan and Zolman 2010), PEX6 (Zolman and Bartel 2004), PEX7 (Woodward and Bartel 2005; Ramón and Bartel 2010), PEX10 (Schumann et al. 2003; Sparkes et al. 2003), PEX12 (Fan et al. 2005; Mano et al. 2006), PEX13 (Mano et al. 2006; Boisson-Dernier et al. 2008; Ratzel et al. 2011), PEX14 (Hayashi et al. 2000), PEX22 (Zolman et al. 2005), and PEX26 (APEM9, Goto et al. 2011). Analysis of these mutants has revealed and confirmed that peroxisomes are important for seedling establishment following germination, lateral root development, photorespiration, jasmonate biosynthesis, and IBA β-oxidation to IAA (reviewed in Hayashi and Nishimura 2006).

Because certain peroxisomal β-oxidation enzymes are required for embryogenesis (Rylott et al. 2003, 2006), any peroxins directly or indirectly needed to import these enzymes into peroxisomes also are expected to be required for embryogenesis. Indeed, null alleles of the ring-finger peroxin genes (*PEX2*, *PEX10*, *PEX12*) and of *APEM9*, the plant *PEX26* equivalent, confer embryonic lethality (Hu et al. 2002; Schumann et al. 2003; Sparkes et al. 2003; Fan et al. 2005; Goto et al. 2011). Combining *pex5-1* with *pex7-1* confers incompletely penetrant embryonic defects (Woodward and Bartel 2005), and combining *pex5-10* or *pex5-1* with the stronger *pex7-2* allele confers embryonic lethality (Ramón and Bartel 2010). Moreover, a *PEX13* null allele confers lethal gametophytic defects (Boisson-Dernier et al. 2008). Whether this early requirement applies to all plant peroxins is not known because surprisingly few *Arabidopsis* peroxin mutants with confirmed null phenotypes have been described. Other reported T-DNA insertion alleles disrupting *PEX*

genes are either in the 5′ UTR and confer only weak defects, as in *pex7-1* (Woodward and Bartel 2005; Ramón and Bartel 2010), *pex13-1* (Ratzel et al. 2011), and *pex22-1* (Zolman et al. 2005), or do not completely abolish function despite insertion in a central exon, as in *pex5-10* (Khan and Zolman 2010; Ramón and Bartel 2010). *pex* mutants isolated using forward genetic screens for IBA resistance, sucrose dependence, or aberrant peroxisome morphology are of course viable, as are knockdown lines generated using RNAi (Nito et al. 2007), but none are demonstrated null alleles. Consistent with an incomplete disruption, *pex2* (*ted3* allele, Hu et al. 2002), *pex4-1* (Zolman et al. 2005), *pex5-1* (Zolman and Bartel 2004), *pex6-1* (Zolman and Bartel 2004), *pex7-2* (Ramón and Bartel 2010), *pex12* (*apm4* allele, Mano et al. 2006), and *pex26* (*apem9-1* allele, Goto et al. 2011) all are missense alleles. Although the *apm3* allele of *pex13* is a nonsense allele, it occurs late in the coding sequence (codon 263 out of 304) and confers only a partial loss of PEX13 function (Mano et al. 2006). This notable under-representation of nonsense alleles and splice site mutations in the extensive collection of *Arabidopsis pex* mutants isolated through forward-genetic screens is consistent with the possibility that complete loss of these peroxins, like the ring finger peroxins, PEX13, and PEX26, may confer embryonic or gametophytic lethality. Moreover, the absence of confirmed T-DNA alleles within the coding sequence of many *PEX* genes hints at an essential role for additional peroxins in gametogenesis, as insertions preventing female gametogenesis may be missing from T-DNA collections (Li et al. 2006).

Our analysis of a *pex14* allelic series provides an apparent exception to the pattern of essential *PEX* genes in *Arabidopsis* and extends the previous analysis of the *ped2* allele of *pex14* that was isolated in the Landsberg accession (Hayashi et al. 2000). Our data confirm the demonstrated role for PEX14 in efficient import of both PTS1 and PTS2 cargo into peroxisomes (Fig. 6; Hayashi et al. 2000), but do not reveal an essential role for PEX14 in importing matrix proteins after the early seedling stage nor an essential role for PEX14 in gametophytic or embryonic development. Although none of our *pex14* alleles accumulated detectable full-length PEX14 protein (Fig. 1e), the *pex14-3* allele displayed the weakest defects in most assays of peroxisomal function (Figs. 2, 3, 4, 5), suggesting that the truncated pex14-3 protein detected in this mutant (Fig. 1e) retained some function. Like the *ped2* allele (Hayashi et al. 2000), *pex14-1* and *pex14-4* are disrupted midway through *PEX14*. The *pex14-2* allele, which is disrupted by a T-DNA insertion early in the *PEX14* coding sequence and lacked detectable *PEX14* mRNA and PEX14 protein (Fig. 1), conferred defects of similar severity to the *pex14-1* and *pex14-4* alleles in physiological assays but appeared to have a slightly stronger block than *pex14-1* in GFP-ICL import into seedling peroxisomes (Fig. 6a), consistent with the possibility that *pex14-1* (and *pex14-4*) may retain slight residual PEX14 function. Indeed, we detected low levels of a truncated pex14-1 protein product in the *pex14-1* mutant (Fig. 1e). Our observations that *pex14* PTS2 processing defects (Figs. 5, 6) and PTS2 import defects (Figs. 6, 7) grew less severe as plants matured and accumulated PEX13 (Fig. 7) are consistent with the possibility that residual inefficient import in the absence of PEX14 gradually transfers cargo into *pex14* peroxisomes and this efficiency may improve as receptor complexes can shift towards PEX13-based import.

Interestingly, even the most severe *pex14* mutants were less IBA resistant (Fig. 2) and displayed more moderate PTS2 processing defects (Fig. 5) than either the *pex5-1* missense allele (Zolman et al. 2000) or the *pex5-10* T-DNA insertion allele (Zolman et al. 2005), suggesting that loss of PEX14 rendered a less complete block in peroxisome function than partial loss of PEX5 function. The same relative order of severity among the *pex5* and *pex14* alleles was not observed in all phenotypes, however; the *pex14* mutants displayed sucrose dependence intermediate between the strong sucrose dependence of *pex5-10* and the weak sucrose dependence of *pex5-1* (Fig. 3), and *pex14* growth defects were more similar to *pex5-10* than to *pex5-1*, which resembles wild type as a mature plant (Fig. 4; Ramón and

Bartel 2010). The *pex5-10* allele confers strong physiological and PTS2 processing defects and severely blocks both PTS1 and PTS2 import pathways (Zolman et al. 2005; Khan and Zolman 2010; Ramón and Bartel 2010), whereas *pex5-1* displays severe PTS2 import defects but imports PTS1 proteins normally (Woodward and Bartel 2005; Ramón and Bartel 2010). The *pex14* mutants displayed severe PTS1 import defects combined with more moderate PTS2 import defects (Fig. 6). The contrasting physiological and molecular defects of *pex5-1* and *pex14* mutants suggest that one or more PTS2 proteins limit IBA responsiveness, whereas PTS1 import may limit fatty acid β-oxidation and growth in the light.

Together, our physiological and molecular analyses suggest that PEX14 augments but ultimately is dispensable for matrix protein import in *Arabidopsis*. Supporting this possibility is the observation that *pex14* alleles are the most frequently recovered *pex* mutants in our forward genetic screens for reduced peroxisome function. Moreover, the lesions in these mutants (e.g., premature termination of the PEX14 polypeptide) are suggestive of severe loss of function, whereas other *pex* mutants that have emerged from these screens are generally missense alleles. Additionally, our most severe allele lacks detectable *PEX14* mRNA and PEX14 protein (Fig. 1d, e), consistent with the possibility that *pex14-2* is a null allele.

The requirement for PEX14 in matrix protein import appears to vary by organism. PEX14 is reported to be essential for matrix protein import in some organisms, including humans. For example, fibroblasts from a patient homozygous for a nonsense mutation in *PEX14* two codons beyond the premature stop in *Arabidopsis pex14-1* completely lack PTS1 and PTS2 import, as monitored using GFP reporters (Shimozawa et al. 2004). However, there are indications that PEX14 may not be essential for matrix protein import in all systems. Although a *Hansenula polymorpha pex14Δ* strain overexpressing PEX5 remains unable to utilize methanol as a carbon source, indicating peroxisome function is not fully restored, PEX5 overexpression partially restores a subset of PTS1 proteins to the peroxisome matrix, indicating that some PTS1 import can occur without PEX14 (Salomons et al. 2000).

In *Arabidopsis*, PEX14, but not PEX13, binds PEX5, whereas PEX13, but not PEX14, binds PEX7 (Nito et al. 2002; Mano et al. 2006), and PEX5 binds PEX7 (Nito et al. 2002; Ramón and Bartel 2010). The observations that an *Arabidopsis pex13* null allele confers gametophyte lethality and completely disrupts GFP-PTS1 import into pollen peroxisomes (Boisson-Dernier et al. 2008) suggests that PEX13 plays an essential role in matrix protein import that cannot be filled by PEX14. Moreover, the IBA resistance, sucrose dependence, and PTS2 processing defects of *pex14-2* are all markedly enhanced by a reduction in *PEX13* expression that alone does not notably impair peroxisome function (Ratzel et al. 2011). The increased severity of PTS1 and PTS2 import defects in very young versus older *pex14* seedlings may reflect the reduced PEX13 levels in younger *pex14* seedlings (Fig. 7).

How might matrix proteins be imported without PEX14? It is possible that the interdependence of the PEX5 and PEX7 receptors provides a partially redundant import pathway that reduces the importance of PEX14 versus PEX13 in *Arabidopsis* compared to organisms such as yeast, in which the receptors independently deliver cargo. Perhaps cargo-laden PEX5-PEX7 complexes normally are targeted to peroxisomes through simultaneous or sequential PEX5-PEX14 and PEX7-PEX13 interactions. When PEX14 is disrupted, PTS2-PEX7 complexes may be inefficiently imported via PEX13. Furthermore, PEX7 docking with PEX13 also may allow PTS1 import via PEX7-PEX5 interactions. Supporting the possibility that PEX7 can contribute to PTS1 import in *Arabidopsis*, we recently found that PTS1 import is impaired in light-grown *pex7* seedlings (Ramón and Bartel 2010). Regardless of the mechanism by which residual peroxisome import is occurring in

Arabidopsis pex14 mutants, our results reinforce the idea that although a core set of peroxins are conserved among many eukaryotes, certain aspects of peroxisome biogenesis mechanisms have diversified in particular lineages.

Materials and methods

Plant materials and growth conditions

Plants were grown in soil (Metromix 200; Scotts, Marysville, OH) at 22°C under continuous illumination by cool-white fluorescent bulbs (Sylvania, Danvers, MA). Plants grown aseptically were plated on PN (plant nutrient medium) (Haughn and Somerville 1986) or PNS (plant nutrient medium supplemented with 0.5% [w/v] sucrose) and solidified with 0.6% (w/v) agar, either alone or supplemented with hormones (from 0.1-, 1.0-, 10-, or 100-mM stocks in ethanol) or kanamycin (from a 25-mg/mL stock). Plates were incubated under yellow long-pass filters to slow the breakdown of indolic compounds (Stasinopoulos and Hangarter 1990) unless indicated otherwise.

Mutants were all in the Columbia (Col-0) accession. *pex5-1* (Zolman et al. 2000), *pex5-10* (Zolman et al. 2005), *pex6-1* (Zolman and Bartel 2004), *pex4-1 pex22-1* (Zolman et al. 2005), and *pex7-2* (Ramón and Bartel 2010) were described previously. *pex14-2* and *pex14-3* were isolated from SALK_007441 and SALK_072373 seeds, respectively, generated by the Salk Institute Genomic Analysis Laboratory, La Jolla, CA (Alonso et al. 2003) and obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). Segregating *pex14-2* and *pex14-3* seeds were plated on PNS supplemented with 12 μg/mL kanamycin, and resistant seedlings were transferred to soil. Plants were genotyped by amplifying genomic DNA from *pex14-2* plants with the primers PED2-9 (5′-GCTTGCTGAACCTCATTAGCAGGCTTAGTAGCC-3′) and LB1-SALK (5′-CAAACCAGCGTGGACCGCTTGCTGCAACTC-3′), which yields an ~500-bp product, or from *pex14-3* plants with primers PED2-6 (5′-GGCAAACCTCATAAAGTATCAATAACCCC-3′) and LB1-SALK, which yields an ~500-bp product. These amplicons were sequenced directly with the LB1-SALK primer to determine the precise location of the T-DNA insert in the mutants.

pex14-1 was isolated from the progeny of Col-0 plants transformed with a T-DNA based cDNA library (LeClere and Bartel 2001) as an IBA-response mutant as previously described (Zolman et al. 2000). Segregation analysis indicated that the T-DNA was not linked to the lesion conferring IBA resistance.

pex14-4 was isolated as a sucrose-dependent IBA-resistant mutant in the Col-0 accession. Twelve pools of ethyl methanesulfonate-mutagenized seed (~72,000 M_2 seeds total) were plated on PN and incubated for 1 day in the light followed by 4 days in darkness. Ungerminated seeds and seedlings with short hypocotyls were transferred to PNS plates and incubated under continuous illumination. After 4 days, expanded seedlings (putative sucrose-dependent mutants) were transferred to a PNS plate supplemented with 10 μM IBA. After another 4–10 days under yellow-filtered light, seedlings that failed to produce abundant lateral roots (putative IBA-resistant mutants) were transferred to soil for seed production. Sucrose dependence and IBA resistance were retested in subsequent generations.

pex14-1 was outcrossed to Wassilewskija (Ws) and *pex14-4* was outcrossed to Landsberg *erecta* (L*er*) *tt4* for recombination mapping. DNA was isolated (Celenza et al. 1995) from IBA-resistant or sucrose-dependent F_2 plants, and mutants were mapped using published PCR-based markers (Konieczny and Ausubel 1993; Bell and Ecker 1994; Davies et al. 1999; Zolman et al. 2001). A candidate gene (*PEX14/PED2; At5g62810*) within the mapping

interval was examined for changes by directly sequencing (LoneStar Labs, Houston, TX) the PCR-amplified *PEX14* gene. Subsequent determination of the *pex14-1* genotype was performed by amplifying genomic DNA with the primers PED2-3 (5′-GTCGTTGGCTGAATATTTTGTTCGGC-3′) and PED2-4 (5′-GTGGCAAGTAAGACCCTAAAGTGAAC-3′), which yields an 1,170-bp product with two *Bcl*I sites in Col-0 and three sites in *pex14-1*. Determination of the *pex14-4* genotype was performed by amplifying genomic DNA with the primers PED2-7 (5′-CAGGGCAATCCAACAACATCCCAA-3′) and PED2-8 (5′-TTGAAGGCTTCTCTCCTCTCTGGA-3′), which yields a 601-bp product with one *Bsa*BI site in Col-0 and two sites in *pex14-4*.

Phenotypic analyses

The *pex14* mutants were each backcrossed to Col-0 at least once prior to analysis. All assays were conducted at least twice with similar results. Seeds were surface-sterilized (Last and Fink 1988) and stratified for 24–48 h prior to plating. In root elongation assays on PN or PNS, seedlings were grown for 8 days and removed from the agar, and the length of the primary root was measured. In root elongation assays on IBA, stratified (24 h) seeds were allowed to germinate in one-sixth liquid PN and 0.5% sucrose for 24 h in white light at 22°C prior to plating on PNS plates supplemented with IBA or the equivalent volume of ethanol (mock). In lateral root assays, seedlings were grown on PNS for 4 days, transferred to PNS or PNS supplemented with 10 μM IBA or 70 nM NAA and grown for an additional 4 days, after which the length of the primary root was measured and lateral roots emerged from the main root were counted. For hypocotyl elongation assays, seeds were plated on PN or PNS and incubated for 1 day in white light to induce germination, followed by 5 days in the dark, after which seedlings were removed from the agar and hypocotyl lengths were measured.

RNA gel-blot analysis

RNA was isolated from 10-day-old light-grown seedlings using the RNeasy kit (Qiagen, Valencia, CA) using the standard extraction protocol. Total RNA was subjected to RNA gel-blot analysis as previously described (Dugas and Bartel 2008). Digoxigenin-labeled probes were amplified using a PCR DIG Probe Synthesis Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions using the primers PEX14-1 (5′-AGAGGCTACTAAGCCTGC-TAATGA-3′) and PEX14-2 (5′-ATGTTGCTGTTCTG TTTCTTCTTG-3′).

Immunoblot analyses

Protein was extracted from seedlings grown under continuous white light on PNS, separated using SDS–PAGE, and electroblotted to HyBond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Zolman and Bartel 2004). After transfer, the membranes were air dried, blocked for 1 h in 8% non-fat dry milk in TBS-T (blocking buffer), and incubated overnight at 4°C with primary antibodies diluted in blocking buffer: rabbit α-GFP (1:300 dilution, BD Biosciences 8372-2), rabbit α-ICL (1:2,000 dilution, Maeshima et al. 1988), rabbit α-MLS (1:25,000 dilution, Olsen et al. 1993), rabbit α-PEX5 (1:100 dilution, Zolman and Bartel 2004), rabbit α-PEX6 (1:1,000 dilution, Ratzel et al. 2011), rabbit α-PEX7 (1:800 dilution, Ramón and Bartel 2010), rabbit α-PEX13 (1:500 dilution, Mano et al. 2006), rabbit α-PEX14 (1:2,500 dilution, Lingard and Bartel 2009), rabbit α-PMDH2 (1:2,000 dilution, Pracharoenwattana et al. 2007), rabbit α-thiolase (PED1 isoform, 1:10,000 dilution, Lingard et al. 2009), mouse α-complex V subunit α (1:2,000 dilution, MitoScience MS507), or mouse α-HSC70 (1:4,000–1:20,000 dilution, StressGen Bioreagents SPA-817), followed by a 1–4 h incubation with horseradish peroxidase-conjugated α-rabbit or α-mouse secondary antibody

(Santa Cruz Biotechnology). Horseradish peroxidase was visualized by incubation with LumiGlo reagent (Cell Signaling Technology, Danvers, MA).

Cell fractionation

Cell fractionation into organellar and cytosolic fractions was as described (Ratzel et al. 2011). Briefly, 500 mg of 3-or 10-day-old light-grown seedlings were homogenized in 1 mL fractionation buffer (150 mM Tris pH 7.6, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 100 mM sucrose, 1× protease inhibitor cocktail [P9559, Sigma]), filtered to give a homogenate (H) fraction, centrifuged at 50×*g* to remove debris, and then centrifuged at 15,300×*g* to give a "soluble" (S) supernatant fraction. The pellet was washed once and resuspended in 40 μL of fractionation buffer to give the "pellet" (P) fraction. Cytosolic fractions contained cytosolic proteins as well as contents of lysed organelles, and pellet fractions contained organellar membranes and contents of intact organelles. Following fractionation, 10 μL of the H (1% total homogenate), S (1% total soluble fraction), and P (25% total pellet) fractions were mixed with 10 μL of NuPAGE 2× loading buffer (Invitrogen) and processed for immunoblot analysis as described above.

Microscopy

Col-0 lines expressing *35S-GFP-PTS1* (Zolman and Bartel 2004), *35S-PTS2-GFP* (Woodward and Bartel 2005), or *ICLp-GFP-ICL* (Lingard et al. 2009) were crossed to *pex14*, and lines homozygous for the mutant and transgene were selected in subsequent generations. Confocal images of cotyledon cells were obtained using a Zeiss multiphoton laser scanning microscope 510 META NLO equipped with a 63× oil-immersion lens. GFP was excited using a 488 nm argon laser. A bandpass emission filter of 500–530 nm was used to detect GFP fluorescence.

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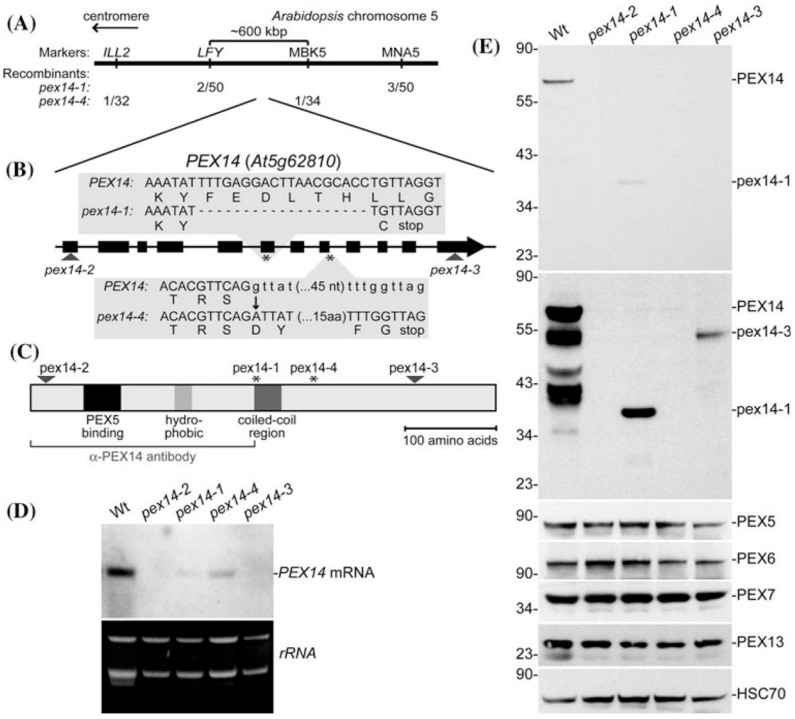

Fig. 1.

A series of *pex14* alleles. **a** Recombination mapping of *pex14-1* and *pex14-4*. Mapping with the PCR-based markers *ILL2* (Davies et al. 1999), *LFY* (Konieczny and Ausubel 1993), MBK5 (Zolman et al. 2001), and MNA5 (Zolman et al. 2001) localized the defects to a region on the bottom of chromosome 5 that contains the *PEX14/PED2* gene. **b** *PEX14* has 12 exons (*thick boxes*) separated by 11 introns (*lines*). The positions of the *pex14-2* and *pex14-3* T-DNA insertions are indicated with *triangles*. The positions of the *pex14-1* and *pex14-4* lesions are marked with *asterisks. pex14-1* has a 19-bp deletion beginning at position 1722 (where 1 is the A of the initiator ATG) resulting in one out-of-frame amino acid followed by an early stop codon. *pex14-4* has a G-to-A mutation at position 2220 which alters the 5′ splice site of the eighth intron, resulting in 19 out-of-frame amino acids followed by an early stop codon. **c** Schematic showing putative domains in PEX14 and the locations of the defects in the four *pex14* alleles. The *bracket* indicates the protein fragment used to generate the α-PEX14 antibody. **d** The four *pex14* alleles display reduced (*pex14-1*, *pex14-4*) or undetectable (*pex14-2*, *pex14-3*) levels of full-length *PEX14* mRNA. RNA extracted from 10-day-old light-grown seedlings was subjected to RNA gel-blot analysis probed with *PEX14* (*top panel*). The *bottom panel* shows the ethidium bromide-stained gel prior to transfer. **e** The four *pex14* alleles lack detectable full-length PEX14 protein, but *pex14-1* and *pex14-3* accumulate pex14 truncation products. Immunoblot of proteins from 8-day-old light-grown seedlings from the indicated lines probed with α-PEX14, α-PEX5, α-PEX6, α-PEX7, α-PEX13, and α-HSC70 (loading control) antibodies. The first and second *panels* are different exposures of the α-PEX14 *panel* to show the presence of the lower molecular mass pex14-1 and pex14-3 products in the longer (second) exposure. Positions of molecular mass markers (in kDa) are indicated on the *left*

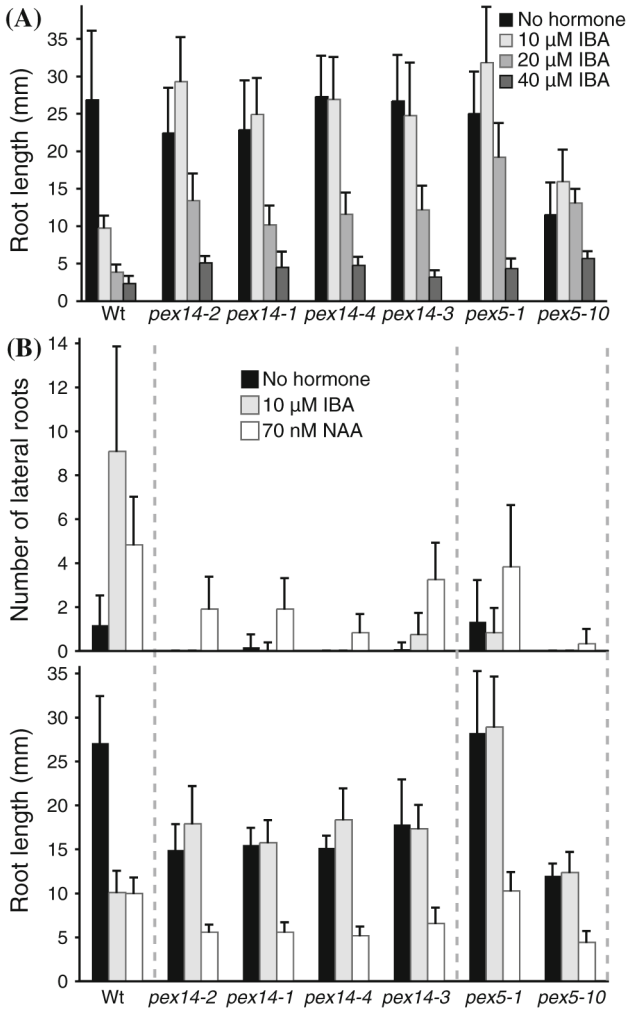

Fig. 2.

pex14 mutant seedlings are IBA resistant. **a** Root elongation on IBA. After 8 days of growth under yellow-filtered light on medium supplemented with the indicated concentration of IBA, seedlings were removed from the agar, and the length of the primary root was measured. *Error bars* represent standard deviations of the means ($n \geq 12$). **b** Lateral root initiation. Lateral roots emerged from the primary root were counted (*upper panel*) and the lengths of the primary roots were measured (*lower panel*) 4 days after transfer of 4-day-old seedlings to unsupplemented medium or medium supplemented with the indicated concentration of IBA or NAA. *Error bars* represent standard deviations of the means ($n \geq 8$)

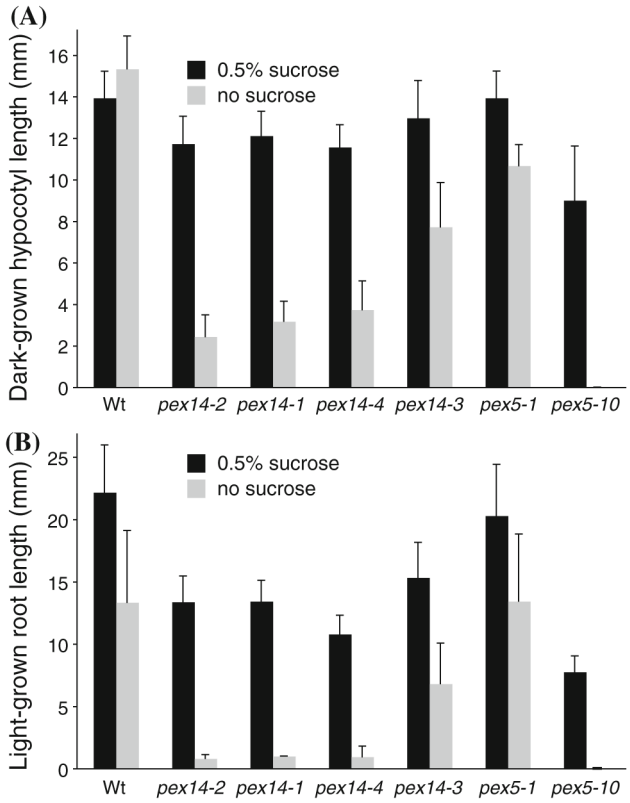

Fig. 3.

pex14 mutant seedlings are sucrose dependent. **a** Hypocotyl elongation in the dark. Seedlings were grown with or without 0.5% sucrose for 1 day under white light and 5 days in darkness, after which hypocotyl lengths were measured. *Error bars* represent standard deviations of the means ($n \geq 14$). **b** Light-grown root elongation. Primary roots were measured after 8 days of growth with or without 0.5% sucrose under white light. *Error bars* represent standard deviations of the means ($n \geq 12$)

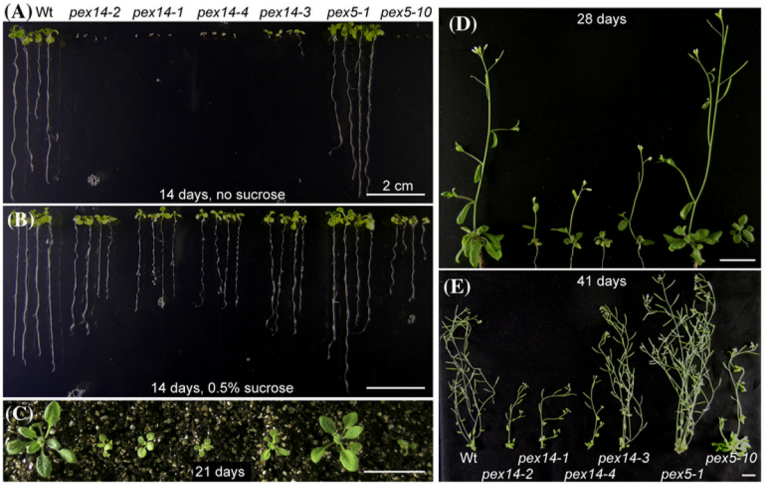

Fig. 4.

pex14 mutant growth defects. **a**, **b** Light-grown 14-day-old wild-type Col-0 (Wt), *pex14*, and *pex5* seedlings on unsupplemented medium (**a**) or medium supplemented with 0.5% sucrose (**b**). **c** 21-day-old plants transferred to soil after 14 days on medium supplemented with 0.5% sucrose. **d** 28-day-old plants transferred to soil after 14 days on medium supplemented with 0.5% sucrose. **e** 41-day-old plants transferred to soil after 14 days on medium supplemented with 0.5% sucrose

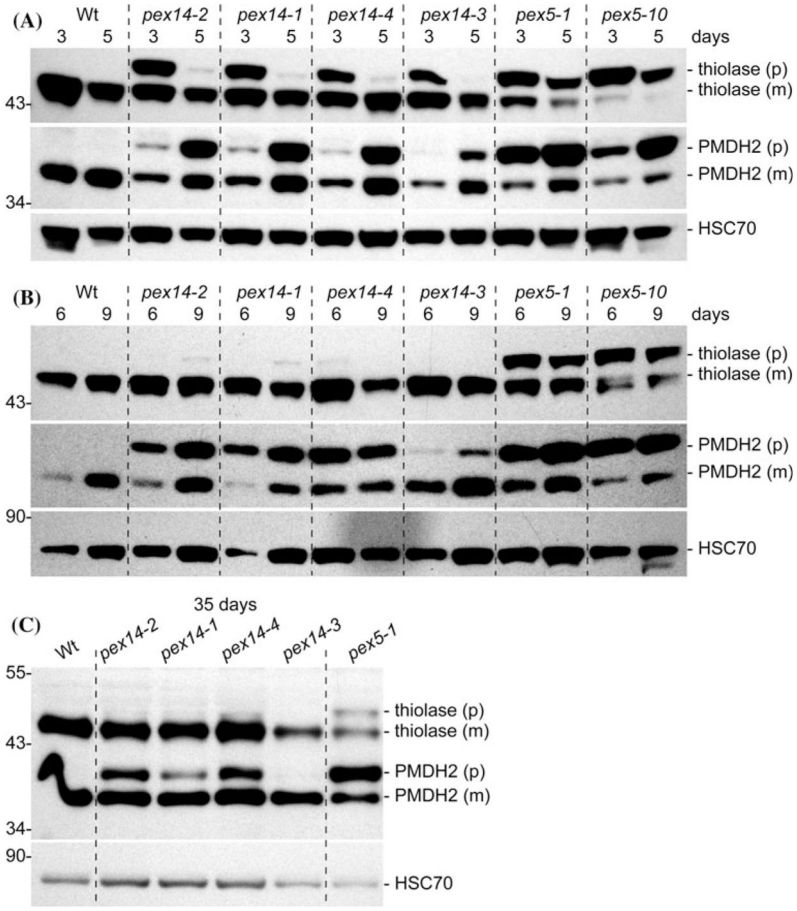

Fig. 5.

pex14 mutants display transient defects in PTS2 protein processing. Immunoblots of proteins prepared from 3- and 5-day-old seedlings (**a**), 6- and 9-day-old seedlings (**b**), or 35-day-old rosette leaves (**c**) probed with α-thiolase and α-PMDH2 antibodies, which recognize precursor (*p*) and mature (*m*) polypeptides, and α-HSC70, a loading control. Positions of molecular mass markers (in kDa) are indicated on the *left*. Upon processing, the N-terminal 4-kDa peptide containing the PTS2 is removed from the 48.5 kDa thiolase precursor and the 37.5 kDa PMDH precursor

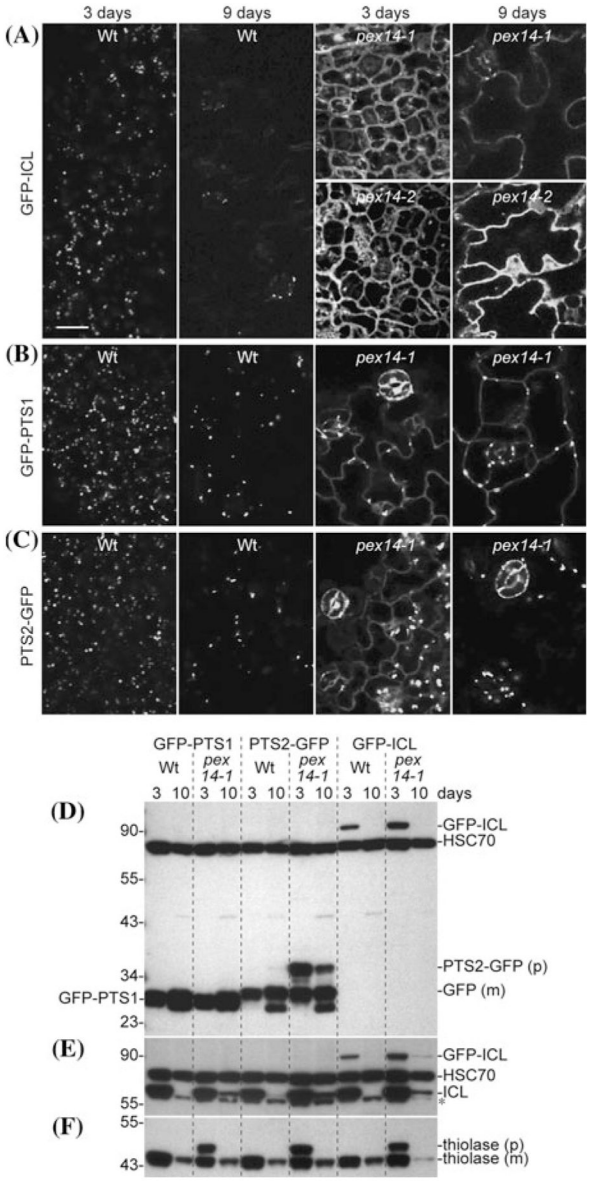

Fig. 6.

pex14 mutants display defects in peroxisome import of both PTS1- and PTS2-tagged matrix proteins. **a** Confocal microscopic images of cotyledon epidermal cells from 3-day- and 9-day-old light-grown seedlings of Col-0 (Wt) and *pex14-1* or *pex14-2* expressing *GFP-ICL* from the *ICL* promoter (Lingard et al. 2009). **b**, **c** Confocal microscopic images of cotyledon epidermal cells from 3-day- and 9-day-old light-grown seedlings of Col-0 (Wt) and *pex14-1* expressing *35S-GFP-PTS1* (**b**) (Zolman and Bartel 2004) or *35S-PTS2-GFP* (**c**) (Woodward and Bartel 2005). In **a**–**c**, the corresponding 3-day-old wild-type and mutant images were acquired with identical microscope settings and the 9-day-old wild-type and mutant images were acquired with identical microscope settings. *Scale bar* 20 μm. **d**–**f** Immunoblot of proteins prepared from 3- and 10-day-old seedlings probed sequentially with α-GFP, α-HSC70, a loading control (**d**), α-ICL (**e**), and α-thiolase (**f**). Precursor (*p*) and mature (*m*) PTS2 proteins (PTS2-GFP and thiolase) are marked on the *right*, and the position of GFP-

PTS1 is marked on the *left*. An *asterisk marks* the position of a protein that cross-reacts with the ICL antibody. Positions of molecular mass markers (in kDa) are indicated on the *left*

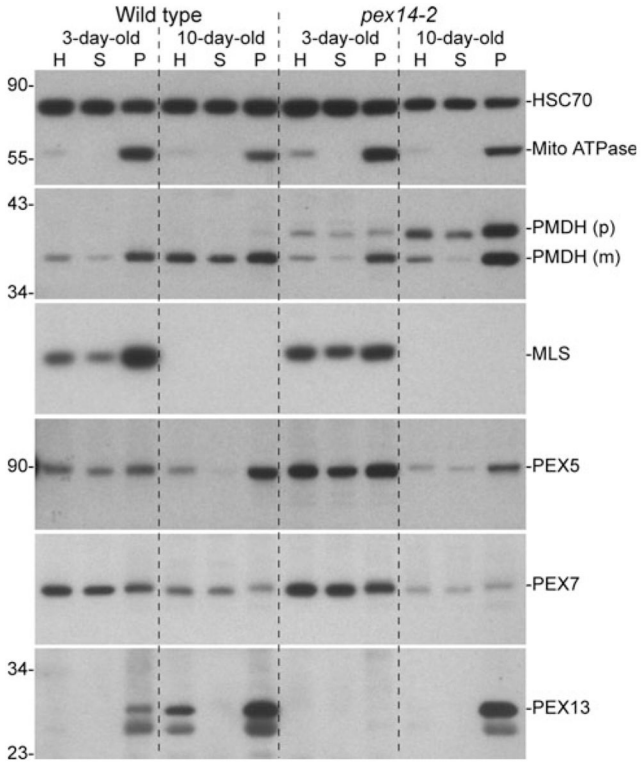

Fig. 7.

pex14-2 displays reduced organellar association of PTS1- and PTS2-cargo proteins and delayed PEX13 accumulation. Extracts from 3- and 10-day-old light-grown wild-type and *pex14-2* seedlings were separated by centrifugation into soluble and organellar pellet fractions. For each sample, 1% of the total homogenate (*H*), 1% of the soluble fraction (*S*), and 25% of the pellet fraction (*P*) were separated using SDS–PAGE and processed for sequential immuno-blotting using the indicated antibodies. HSC70 and the mitochondrial membrane complex V subunit α (mito ATPase) were used as cytosolic and organellar controls, respectively. Precursor (*p*) and mature (*m*) proteins contain or lack the N-terminal PTS2 peptide, respectively. Positions of molecular mass markers (in kDa) are indicated on the *left*