Genetic dissection of peroxisome-associated matrix protein degradation in

*Arabidopsis thaliana*

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Running title: Peroxisomal protein degradation

Key words: organelle quality control, peroxin, peroxisome, PEX6 AAA ATPase, protein degradation

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ABSTRACT

Peroxisomes are organelles that sequester certain metabolic pathways; many of these pathways generate H$_2$O$_2$, which can damage proteins. However, little is known about how damaged or obsolete peroxisomal proteins are degraded. We are exploiting developmentally timed peroxisomal content remodeling in Arabidopsis thaliana to elucidate peroxisome-associated protein degradation. Isocitrate lyase (ICL) is a peroxisomal glyoxylate cycle enzyme necessary for early seedling development. A few days after germination, photosynthesis begins and ICL is degraded. We previously found that ICL is stabilized when a peroxisome-associated ubiquitin-conjugating enzyme and its membrane anchor are both mutated, suggesting that matrix proteins might exit the peroxisome for ubiquitin-dependent cytosolic degradation. To identify additional components needed for peroxisome-associated matrix protein degradation, we mutagenized a line expressing GFP-ICL, which is degraded similarly to endogenous ICL, and identified persistent GFP-ICL fluorescence (pfl) mutants. We found three pfl mutants that were defective in PEROXIN14 (PEX14/At5g62810), a peroxisomal membrane protein that assists in importing proteins into the peroxisome matrix, indicating that proteins must enter the peroxisome for efficient degradation. One pfl mutant was missing the peroxisomal 3-ketoacyl-CoA thiolase encoded by the PEROXISOME DEFECTIVE1 (PED1/At2g33150) gene, suggesting that peroxisomal metabolism influences the rate of matrix protein degradation. Finally, one pfl mutant that displayed normal matrix protein import carried a novel lesion in PEROXIN6 (PEX6/At1g03000), which encodes a peroxisome-tethered ATPase that is involved in recycling matrix protein receptors back to the cytosol. The isolation of pex6-2 as a pfl mutant supports the hypothesis that matrix proteins can exit the peroxisome for cytosolic degradation.
Introduction

Peroxisomes are single membrane bound organelles that house essential metabolic pathways in plants and other eukaryotes. For example, peroxisome biogenesis defects underlie the Zellweger spectrum of human congenital disorders, which often are fatal in infancy (reviewed in Wanders and Waterham 2005). Similarly, peroxisomes are essential for plant embryogenesis and development following germination (reviewed in Hu et al. 2012). The essential role of plant peroxisomes likely reflects the importance of peroxisomal enzymes, which catalyze key steps in photorespiration, fatty acid β-oxidation, jasmonate production, and conversion of the protoauxin indole-3-butyric acid (IBA) to the active auxin indole-3-acetic acid (IAA) (reviewed in Hu et al. 2012).

Peroxisomes import matrix proteins from the cytosol with the assistance of peroxin (PEX) proteins. Most matrix proteins are directed to the peroxisome by a C-terminal peroxisome-targeting signal 1 (PTS1) that binds the cytosolic receptor PEX5 (Keller et al. 1987). PEX5-cargo complexes dock with the PEX13 and PEX14 membrane peroxins (reviewed in Azevedo and Schliebs 2006; Williams and Distel 2006) at the peroxisome membrane. Other matrix proteins use an N-terminal PTS2 to bind the cytosolic receptor PEX7 (Osumi et al. 1991; Swinkels et al. 1991). In plants and mammals, PEX7 depends on PEX5 (Matsumura et al. 2000; Hayashi et al. 2005; Woodward and Bartel 2005) for cargo delivery to the PEX13 and PEX14 docking peroxins (reviewed in Lazarow 2006). After matrix proteins are delivered, yeast PEX5 is ubiquitinated in the peroxisome membrane by the ubiquitin-conjugating enzyme PEX4 and the ubiquitin-protein ligase PEX12 (Platta et al. 2009). Ubiquitinated PEX5 is retrotranslocated to the cytosol with the assistance of the peroxisome-tethered ATPases PEX1 and PEX6 (reviewed in Fujiki et al. 2012; Grimm et al. 2012) to be reused in further rounds of import.

Many metabolic pathways sequestered in peroxisomes produce hydrogen peroxide ($H_2O_2$). For example, $H_2O_2$ is generated by the acyl-CoA oxidases acting in fatty acid β-oxidation (Eastmond et al. 2000b; Adham et al. 2005) and the glycolate oxidases acting in photorespiration (Fahnenstich et al. 2008). $H_2O_2$ can damage proteins (Van Den Bosch et al. ...
but little is known about how damaged or obsolete peroxisomal proteins are degraded. Three possible mechanisms for peroxisomal matrix protein degradation can be envisioned: degradation within the organelle by resident proteases, degradation of the entire organelle via autophagy, or retrotranslocation out of the organelle followed by cytosolic degradation.

Many organelles, including mitochondria and chloroplasts, contain proteases that degrade damaged or misfolded proteins (reviewed in Leidhold and Voos 2007). Several proteases are found in Arabidopsis peroxisomes (Reumann et al. 2004; Helm et al. 2007; Reumann et al. 2007; Lingard and Bartel 2009). For example, DEG15 cleaves PTS2 proteins from their targeting signal after import (Helm et al. 2007; Schumann et al. 2008) and the LON2 ATP-dependent protease is needed for sustained matrix protein import (Lingard and Bartel 2009). Although a fungal LON isoform contributes to degradation of oxidatively damaged peroxisomal matrix proteins (Bartoszewska et al. 2012), no resident peroxisomal proteases have been implicated in matrix protein degradation in plants.

A second possibility for peroxisomal protein degradation is removal of the entire organelle by autophagy or pexophagy, a specialized form of autophagy. For example, yeast use pexophagy to degrade excess peroxisomes by encasing the peroxisome in a membrane for fusion with the vacuole (reviewed in Manjithaya et al. 2010). Although autophagy occurs in Arabidopsis (reviewed in Li and Vierstra 2012), pexophagy has not been reported in plants.

A third potential mechanism for peroxisome-associated protein degradation is modeled after ER-associated protein degradation (ERAD), the process by which misfolded proteins are ubiquitinated and retrotranslocated from the ER lumen to the cytosol for proteasomal degradation (reviewed in Hoseki et al. 2010). Peroxins needed for PEX5 ubiquitination and retrotranslocation resemble ERAD components (Gabaldon et al. 2006; Schluter et al. 2006), suggesting that damaged peroxisomal proteins may be retrotranslocated out of the peroxisome and degraded in the cytosol by the 26S proteasome (Zolman et al. 2005).
Some evidence in Arabidopsis is consistent with a retrotranslocation model for matrix protein degradation. Isocitrate lyase (ICL) and malate synthase (MLS) are peroxisomal glyoxylate cycle enzymes that enable carbon from acetyl-CoA to be utilized in gluconeogenesis, thus providing energy for germinating seedlings (reviewed in Graham 2008). In Arabidopsis, ICL and MLS are degraded a few days after germination (Zolman et al. 2005; Lingard et al. 2009). Mutation of the PEX4 ubiquitin-conjugating enzyme along with PEX22, which tethers PEX4 to the peroxisome (pex4-1 pex22-1; Zolman et al. 2005), partially stabilizes MLS, ICL, and a GFP-ICL translational fusion without markedly impairing matrix protein import (Zolman et al. 2005; Lingard et al. 2009). Stabilization of these glyoxylate cycle enzymes in pex4-1 pex22-1 suggests a role for PEX4-mediated ubiquitination in promoting matrix protein degradation.

To identify additional components necessary for the turnover of damaged or unnecessary peroxisomal proteins, we initiated a forward genetic screen for Arabidopsis mutants exhibiting delayed GFP-ICL degradation. We identified several mutants with prolonged GFP-ICL fluorescence that also stabilized endogenous ICL. Characterization of these mutants confirmed that matrix proteins must enter the peroxisome to be subject to efficient degradation and is consistent with the possibility that damaged or obsolete matrix proteins can exit the peroxisome for cytosolic degradation.

**Materials and Methods**

*Plant materials and growth conditions*

*Arabidopsis thaliana* accession Columbia (Col-0) or Col-0 transformed with ICLp:GFP-ICL, which drives a GFP-ICL fusion protein from the ICL 5' regulatory region (Lingard et al. 2009), was used as wild type (as indicated in figure legends). pex14-1, pex14-2/SALK_007441, pex14-3/SALK_072373, pex14-4 (Monroe-Augustus et al. 2011), ped1-96 (Lingard and Bartel 2009), lon2-2/SALK_043857 (Lingard and Bartel 2009), pex6-1 (Zolman and Bartel 2004), and pex6-1 lines transformed with pBINPEX6, 35S:HsPEX6, and 35S:PEX5 (Zolman and Bartel
2004) were previously described. Prior to phenotypic analyses, pfl7/ped1-7, pfl20/lon2-6, pfl47/pex6-2, pfl49/pex14-6, and pfl175/pex14-5 were backcrossed to wild-type Col-0 at least once. Unidentified mutants (pfl29, pfl99, and pfl106) and the second pex14-5 isolate (pfl164) were characterized using non-backcrossed progeny of original isolates. pex6-2 carrying pBINPEX6, 35S:HsPEX6, or 35S:PEX5 were generated by Agrobacterium tumefaciens-mediated transformation (Clough and Bent 1998) of backcrossed pex6-2 lacking the ICLp:GFP-ICL transgene. Homozygous lines were selected in the progeny of transformants by following resistance to kanamycin (pBINPEX6) or glufosinate ammonium (Basta) (35S:HsPEX6 and 35S:PEX5).

Surface-sterilized seeds were plated on plant nutrient (PN) medium (Haughn and Somerville 1986) supplemented with 0.5% (w/v) sucrose (PNS) and solidified with 0.6% (w/v) agar. Hormone stocks were dissolved in ethanol at 10 or 100 mM and media normalized to the same ethanol content were used as controls. For assays of light-grown seedlings, seeds were stratified for one day, plated on the indicated auxin concentrations, and grown for 8 days at 22° under continuous illumination through yellow long-pass filters, which slow indolic compound breakdown (Stasinopoulos and Hangarter 1990), unless otherwise indicated. For assays with dark-grown seedlings, seeds were stratified for one day, allowed to begin germination in the absence of hormones under yellow light for one day, plated on the indicated media, returned to yellow light for one day, and placed in darkness for 4 or 5 days, after which hypocotyl lengths were measured. For lateral root assays, seeds were stratified for one day, plated on PNS, and grown in yellow light for four days. Four-day-old seedlings were then transferred to a mock or IBA-containing PNS plate and grown under yellow light for four additional days, after which the primary root length was measured and the number of lateral roots that had emerged from the primary root were counted.
Mutant isolation and recombination mapping

Seeds from Col-0 lines transformed with *ICLp:GFP-ICL* (Lingard *et al.* 2009) were mutagenized with ethyl methanesulfonate (EMS, Normanly *et al.* 1997). M2 seeds were surface sterilized (Last and Fink 1988), stratified for 0-1 day, plated on PNS (~1000 seeds per 100 x 100 x 15 mm square plate), and grown in white light. Mutants displaying GFP-ICL fluorescence at 7-9 days were selected using a Leica MZ FLIII fluorescence stereomicroscope equipped for GFP detection, transferred to a fresh PNS plate to recover, and moved to soil for seed production. For retesting, M3 progeny seeds were stratified for 3 days to promote uniform germination and assayed for prolonged fluorescence after 6-7 days of growth under white light. Lines displaying prolonged fluorescence were retained as *persistent GFP-ICL fluorescence (pfl)* mutants.

Mutants isolated from Col-0 carrying the *ICLp:GFP-ICL* construct were outcrossed to Landsberg erecta (Ler) for recombination mapping. F2 seedlings from *pfl47* and *pfl106* outcrosses were screened on PNS for prolonged GFP-ICL fluorescence compared to the unmutagenized parent lines and F3 progeny of mapping plants were confirmed to have prolonged fluorescence. F2 seedlings from *pfl7* and *pfl99* outcrosses were screened for sucrose dependence. F2 seedlings from *pfl20* and *pfl29* outcrosses were screened for IBA-resistant root elongation. DNA was isolated from individuals in the mapping populations showing the mutant phenotype and assayed using published and newly developed PCR-based polymorphic markers (Table 1).

Immunoblot analysis

To avoid complications in assessing the timing of ICL or MLS degradation that would arise if a mutant exhibited delayed germination, time course immunoblots used seedlings that had germinated within 24 hours after plating. Protein was extracted from seedlings grown under continuous white light on PNS for the indicated number of days. To extract protein, frozen tissue was ground with a pestle and an equal volume of 2x loading buffer (Invitrogen, Carlsbad, CA) was added. Samples were centrifuged and 20 µL of supernatant was transferred to a fresh tube with 2.1 µL of 0.5 M dithiothreitol and heated at 100°C for 5 minutes. Samples were loaded onto
NuPAGE 10% Bis-Tris gels (Invitrogen) next to prestained protein markers (P7708S, New England Biolabs, Beverly, MA) and Cruz Markers (Santa Cruz Biotechnology, Santa Cruz, CA). After electrophoresis, proteins were transferred for 30 minutes at 24 V to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using NuPAGE transfer buffer (Invitrogen). After transfer, membranes were rocked for 1 h at 4°C in blocking buffer (8% non-fat dry milk, 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20), 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-HPR (1:1000 dilution, Agrisera AS11 1797 or Kleczkowski and Randall 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-PEX14 (1:2,500 dilution, Agrisera AS08 372), rabbit α-PMDH2 (1:2,000 dilution, Pracharoenwattana et al. 2007), rabbit α-thiolase (PED1 isoform, 1:10,000 dilution, Lingard et al. 2009), or mouse α-HSC70 (1:20,000–1:30,000 dilution, StressGen Bioreagents SPA-817), followed by a 4 h incubation with horseradish peroxidase-linked goat α-rabbit or α-mouse IgG secondary antibody (1:5000 dilution in blocking buffer, Santa Cruz Biotechnology, SC2030 or SC-2031). Horseradish peroxidase was visualized by incubation with LumiGlo reagent (Cell Signaling Technology, Danvers, MA) or WesternBright ECL reagent (Advansta, Menlo Park, CA).

**RNA analysis**

RNA was isolated from 8-d-old light-grown Col-0 and pex14-6 seedlings using the TRI Reagent RNA Extraction method according to the instructions of the manufacturer (Sigma, St. Louis, MO) and dissolved in DEPC-treated water. cDNA was synthesized from RNA using a 3’ gene-specific primer (PED2-6; Table 2) and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The pex14-6 cDNA was PCR-amplified across the exon 1-2 junction using PED2-1 and PED2-4 primers (Table 2) and sequenced using PED2-1.
**Confocal microscopy**

Four-day-old seedlings carrying *ICLp:GFP-ICL* (Lingard *et al.* 2009) were mounted in water under a cover glass. Images were collected using a Carl Zeiss LSM 710 laser scanning confocal microscope equipped with a Meta detector. Samples were imaged through a 40x oil immersion objective after excitation with a 488-nm argon laser; GFP emission was collected between 494 and 560 nm. Each image is an average of 8 exposures using a 70-µm pinhole, corresponding to a 1.8 µm optical slice.

**Sequencing and genotyping of mutant lesions**

Candidate genes in mapping intervals were PCR-amplified from mutant genomic DNA using primers listed in Table 2. Amplicons were purified using Zymo PCR purification kit (Zymo Research, Irvine, CA) and sequenced directly (Lone Star Labs, Houston, TX) with the primers used for amplification.

The *PEX14* gene (*At5g62810*) was amplified from *pfl49*, *pf164*, and *pf175* genomic DNA using three oligonucleotide pairs (Table 2). The resulting overlapping fragments covered the gene from 75 bp upstream of the translation start site to 29 bp downstream of the stop codon. The lesion identified in *pfl49* changed *PEX14* G73 (where 1 is the first nucleotide of the initiator codon) to an A, which altered a splice site. The lesion identified in *pfl164* and *pfl175* (which are likely siblings as they were isolated from the same pool of mutagenized seeds) changed *PEX14* G904 (where 1 is the first nucleotide of the initiator codon) to an A, which changed Trp152 to a stop codon.

The *PED1* gene (*At2g33150*) was amplified from *pfl7* genomic DNA using four oligonucleotide pairs (Table 2). The resulting overlapping fragments covered the gene from 72 bp upstream of the translation start site to 274 bp downstream of the stop codon. The lesion identified in *pfl7* changed G2624 (where 1 is the first nucleotide of the initiator codon) of *PED1* to an A, which altered a splice site.
The *LON2* gene (*At5g47040*) was amplified from *pfl20* genomic DNA using six oligonucleotide pairs (Table 2). The resulting overlapping fragments covered the gene from 1307 bp upstream of the translation start site to 625 bp downstream of the stop codon. The lesion identified in *pfl20* changed G2809 (where 1 is the first nucleotide of the initiator codon) of *LON2* to an A, which created an amino acid change (Gly445Arg) and destroyed an *MnlI* restriction site.

The *PEX6* gene (*At1g0300*) was amplified from *pfl47* genomic DNA using five oligonucleotide pairs (Table 2). The resulting overlapping fragments covered the gene from 151 bp upstream of the translation start site to 346 bp downstream of the stop codon. The lesion identified in *pfl47* changed C1156 (where 1 is the first nucleotide of the initiator codon) of *PEX6* to a T, which created an amino acid change (Leu328Phe).

Identified mutations were followed in the progeny of crosses using PCR amplification with the primers listed in Table 3 followed by digestion of the resultant amplicons with the restriction enzymes indicated in Table 3. The *pex6-1* mutation was followed as previously described (Zolman and Bartel 2004).

**Results**

**Screening for mutants with stabilized GFP-ICL**

ICL is a peroxisomal matrix protein that is degraded a few days after Arabidopsis seedling germination (Zolman *et al.* 2005; Lingard *et al.* 2009). GFP-ICL driven from the endogenous *ICL* promoter is degraded with similar kinetics as unmodified ICL; GFP-ICL fluorescence, like ICL protein, is no longer apparent 5 to 6 days after plating (Lingard *et al.* 2009). To isolate mutants with defects in peroxisome-associated protein degradation, we screened for mutants that exhibited prolonged GFP-ICL fluorescence. We mutagenized lines carrying the *ICLp:GFP-ICL* construct with EMS and screened ~44,500 of the resulting M₂ seedlings for GFP-ICL fluorescence that remained visible 7-9 days after plating. We selected 175 putative mutants exhibiting prolonged fluorescence. Of these, 105 died or were infertile, 49 did not display prolonged fluorescence in the M₃ generation, and 21 appeared to prolong GFP-ICL
fluorescence in the M₃ generation. We used confocal microscopy to examine GFP-ICL localization in several of these persistent GFP-ICL fluorescence (pfl) mutants. As summarized in Table 4, we found three with extensive cytosolic GFP-ICL fluorescence (Figure 1, F and I), three with punctate GFP-ICL fluorescence similar to the unmutagenized parent (Figure 1, C, E, and G), and three with partially punctate and partially cytosolic GFP-ICL fluorescence (Figure 1, B, D, and H). In addition to these matrix protein localization defects, we observed some aberrations in peroxisome appearance in the mutants. For example, peroxisomes appeared clustered in pfl7 and pfl106 (Figure 1B and H), larger in pfl20 (Figure 1C), and smaller in pfl29 (Figure 1D) compared to wild type (Figure 1A).

**Peroxisome function in persistent GFP-ICL fluorescent (pfl) mutants**

Because defects in peroxisomal matrix protein import often are accompanied by defects in peroxisomal metabolism, we tested peroxisome function in the pfl mutants using sucrose dependence and IBA resistance assays, which indirectly assess the efficiency of peroxisomal β-oxidation. Peroxisomal fatty acid β-oxidation provides energy for early seedling development prior to the onset of photosynthesis. Certain peroxisome-defective mutants, such as pxa1-1 (Zolman et al. 2001), arrest or develop slowly following germination because fatty acids are inefficiently metabolized. These defects can be partially bypassed by providing a fixed carbon source, such as sucrose, in the growth medium (Hayashi et al. 1998; Zolman et al. 2000). Four mutants (pfl7, pfl99, pfl164, and pfl175) displayed clear sucrose-dependent root elongation in the light (Figure 2A) and/or hypocotyl elongation in the dark (Figure 2B), suggesting inefficient β-oxidation of stored fatty acids, which could result from defects in peroxisome biogenesis or β-oxidation enzymes.

We also compared peroxisome function in the pfl mutants using IBA resistance assays. Because the protoauxin IBA is imported into peroxisomes and converted into the active auxin, IAA (reviewed in Strader and Bartel 2011), IBA application reduces primary root elongation (Zolman et al. 2000), inhibits hypocotyl elongation in dark-grown seedlings (Strader et al. 2008),
and promotes proliferation of lateral roots in light-grown seedlings (Zolman et al. 2000; Zolman et al. 2001; Zolman et al. 2007; Zolman et al. 2008). When IBA-to-IAA conversion is impaired, the auxin effects of IBA are diminished (Strader et al. 2010). Similarly, peroxisomal β-oxidation of the IBA analog 2,4-dichlorophenoxybutyric acid (2,4-DB) to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) generates auxin phenotypes (Hayashi et al. 1998). Typical peroxin mutants, such as pex6-1 (Zolman and Bartel 2004), display resistance to the inhibitory effects of IBA (and 2,4-DB) on root elongation in the light and hypocotyl elongation in the dark (Zolman and Bartel 2004; Strader et al. 2011). We found that seven pfl mutants (pfl7, pfl20, pfl29, pfl47, pfl49, pfl164, and pfl175) were IBA (and 2,4-DB) resistant in root (Figure 2A) and/or hypocotyl elongation (Figure 2B), suggesting inefficient β-oxidation of IBA to IAA (and 2,4-DB to 2,4-D) in these mutants.

**Mutations in the gene encoding the PEX14 receptor-docking peroxin stabilize GFP-ICL**

The three persistent GFP-ICL fluorescence mutants with predominantly cytosolic GFP-ICL (pfl49, pfl164, and pfl175; Figure 1 and Table 4) also displayed IBA and 2,4-DB resistance (Figure 2, A and B), consistent with inefficient matrix protein import. Because peroxins facilitate peroxisomal matrix protein import, we examined levels of several peroxins in the pfl mutants using immunoblot analysis. We found normal levels of PEX5, PEX6, and PEX7, but reduced levels of full-length PEX14 protein (Figure 2C) in all three mutants with predominantly cytosolic GFP-ICL (Figure 1, F and I). Upon sequencing the PEX14 gene (At5g62810) in these mutants, we identified two novel point mutations, which we renamed pex14-5 (pfl164 and pfl175) and pex14-6 (pfl49). pex14-5 changes a Trp to a stop codon in the fourth exon (Figure 3A). Overexposure of an anti-PEX14 immunoblot did not reveal PEX14 protein in pex14-5 seedlings (Figure 3D), suggesting that pex14-5 is a null allele. pex14-6 harbors a mutation in the last nucleotide of exon 1, which would change Glu25 to Lys and disrupt a slice site (Figure 3A), and accumulates a small amount of nearly full-length PEX14 protein (Figure 3D). We isolated RNA from the pex14-6 mutant and determined the major
pex14-6 splice product uses a cryptic 5′-donor site in the 5′-UTR, thereby skipping the first exon of PEX14.

We compared these two new pex14 alleles to several previously characterized pex14 alleles (Monroe-Augustus et al. 2011) and found that the new alleles conferred similar IBA- and 2,4-DB resistance in both dark- and light-grown seedlings (Figure 3, B and C) along with transient defects in removal of the PTS2-containing presequence from the matrix protein thiolase (Figure 3E). Unlike other pex14 alleles, the pex14-6 allele was not dependent on sucrose in the dark (Figure 3B), suggesting that the low level of pex14-6 protein detected in this mutant (Figure 3D) retained partial PEX14 function.

To assess whether disruption of PEX14 stabilizes endogenous peroxisomal matrix proteins, we compared ICL stability in wild type, pex14-5, pex14-6, and pex14-2, a previously described pex14 null allele (Monroe-Augustus et al. 2011). We found that ICL protein was stabilized in all three pex14 alleles compared to wild type (Figure 3E). Our recovery of pex14 alleles as persistent GFP-ICL fluorescence mutants suggests that impaired peroxisome matrix protein import prevents access of GFP-ICL and ICL to the peroxisome-associated proteolysis machinery or the factors or conditions needed to target substrates to this machinery.

A mutation in a gene encoding the LON2 peroxisomal protease

The pfl20 mutant displayed punctate GFP-ICL fluorescence in 4-d-old seedlings (Figure 1C). We used the associated phenotype of IBA-resistant primary root elongation (Figure 2A) to map the pfl20 lesion to an interval on the bottom of chromosome 5 (Figure 4) that included the gene encoding the LON2 (At5g47040) peroxisomal ATP-dependent protease (Lingard and Bartel 2009). We sequenced LON2 from pfl20 genomic DNA and found a point mutation in exon 10 (Figure 5A) that changed Gly445 to an Arg residue. The mutated Gly residue is in the AAA-ATPase domain (Figure 5A) between the Walker A and B domains and is invariant in LON isoforms from plants, fungi, bacteria, and mammals. Like other lon2 alleles (Lingard and Bartel 2009), pfl20 displayed moderate resistance to the inhibition of root and hypocotyl elongation by
IBA (Figure 5, B and C), severe resistance to the promotive effects of IBA on lateral rooting (Figure 5D), and PMDH PTS2 processing defects (Figure 5E). We renamed this mutant lon2-6.

Because LON2 is a peroxisomal protease (Ostersetzer et al. 2007), it was a candidate for participation in peroxisome-associated protein degradation. However, previously characterized lon2 T-DNA insertion alleles do not dramatically stabilize ICL or MLS (Lingard and Bartel 2009). To examine whether peroxisomal matrix proteins were stabilized in the lon2-6 mutant, we compared ICL stability in wild type, lon2-6, and lon2-2, a previously characterized (Lingard and Bartel 2009) lon2 allele disrupted by a T-DNA insertion near the 3′ end of the gene (Figure 5A). Indeed, we found that ICL was not stabilized in either lon2 mutant compared to wild type (Figure 5E). Some lon2 alleles display uneven germination (Lingard and Bartel 2009), suggesting that a germination delay of the M2 seedling may explain our original isolation of the pfl20/lon2-6 mutant in the persistent GFP-ICL fluorescence screen.

**Mutations in the PED1 gene encoding a peroxisomal thiolase stabilize GFP-ICL**

pfl7 displayed a combination of peroxisomal and cytosolic GFP-ICL fluorescence (Figure 1B) and the classical peroxisome-defective phenotypes of IBA and 2,4-DB resistant root and hypocotyl elongation and sucrose-dependent seedling development (Figure 2, A and B). We used the sucrose dependence phenotype to map the pfl7 lesion to a region of chromosome 2 that included PED1 (At2g33150; Figure 4), which encodes a peroxisomal 3-ketoacyl-CoA thiolase also known as KAT2 that is implicated in fatty acid, IBA, and 2,4-DB β-oxidation (Hayashi et al. 1998; Germain et al. 2001). In addition, we did not detect thiolase protein on immunoblots of pfl7 seedling extracts (Figure 2C). We sequenced PED1 from pfl7 DNA and found a point mutation in the first nucleotide of intron 10 that is predicted to disrupt PED1 splicing (Figure 5A). The nature of the lesion was consistent with the lack of full-length thiolase (PED1) protein detected in immunoblots of pfl7 seedling extracts (Figures 2C and 5E), and we renamed pfl7 as ped1-7.
We found that *ped1-7* displayed β-oxidation defects similar in severity to *ped1-96* (Figure 5, B-D), a previously isolated *ped1* null allele (Lingard and Bartel 2009). However, matrix protein import defects have not been reported for *ped1-96* (Lingard and Bartel 2009), the original *ped1* allele (Hayashi et al. 1998), or the *kat2-1* T-DNA insertion allele of *PED1* (Germain et al. 2001). Because *ped1-7* partially mislocalized GFP-ICL to the cytosol (Figure 1B), we examined PTS2 processing in *ped1* mutants. We found that both *ped1-7* and *ped1-96* displayed a slight defect in PTS2 processing of PMDH (Figure 5E), consistent with the slight defect in matrix protein import revealed by the partial mislocalization of GFP-ICL to the cytosol in *ped1-7* (Figure 1B).

To examine whether disruption of the PED1 thiolase stabilizes endogenous peroxisomal matrix proteins, we compared ICL stability in wild type, *ped1-7*, and *ped1-96*. We found that ICL was similarly stabilized in both *ped1* alleles (Figure 5E). To examine whether this stabilization might reflect a developmental delay caused by the reduced fatty acid β-oxidation that would result from reduced thiolase activity, we monitored the timing of appearance of the photorespiration enzyme hydroxypyruvate reductase (HPR) in the *ped1* mutants. In wild-type seedlings, HPR appears during the transition to photosynthetic growth as ICL is degraded (Lingard et al. 2009). We found that HPR appeared in *ped1* mutants with similar timing as in wild type (Figure 5E), suggesting *ped1* developmental delays did not account for the observed ICL stabilization (Figure 5E).

**The PEX6 AAA-ATPase is required for efficient peroxisome-associated degradation**

The *pfl47* mutant displayed normal peroxisomal localization of GFP-ICL (Figure 1E) and normal levels of assayed peroxins (Figure 2C). We used the persistent GFP-ICL fluorescence phenotype to map *pfl47* to a region at the top of chromosome 1 that included the *PEX6* gene (*At1g03000*; Figure 4). Upon sequencing *PEX6* from *pfl47* DNA, we found a point mutation in exon 3 that changed Leu328 to a Phe residue (Figure 6A). We renamed *pfl47* as *pex6-2*. We compared the phenotypes of *pex6-2* to those of *pex6-1*, a different missense allele isolated in a
screen for mutants displaying IBA resistant root elongation that also is sucrose dependent and displays a marked PTS2 processing defect (Zolman and Bartel 2004). Unlike pex6-1, pex6-2 developed normally in the absence of sucrose in the dark (Figure 6B), was only moderately resistant to the inhibitory effects of IBA on hypocotyl (Figure 6B) or root (Figure 6C) elongation, processed the PTS2 proteins thiolase and PMDH nearly normally (Figures 2C and 6E), and displayed a wild-type root length on sucrose-supplemented medium (Figure 6C). Both pex6 alleles displayed clear resistance to the inhibitory effects of 2,4-DB on hypocotyl elongation in the dark (Figure 6B) and to the promotive effects of IBA on lateral root formation in the light (Figure 6D). Moreover, both alleles similarly stabilized ICL and MLS (Figure 6, E and F). Because the pex6-2 phenotypes were not identical to those of pex6-1, we introduced a wild-type genomic copy of PEX6 (Zolman and Bartel 2004) into pex6-2 using Agrobacterium-mediated transformation to ensure that the identified pex6-2 lesion was responsible for the phenotypes observed. We found that this PEX6p:PEX6 construct rescued the 2,4-DB (Figure 7A) and IBA resistance (Figure 7B) of pex6-2 and pex6-1, confirming that the identified pex6-2 lesion caused the peroxisome-defective phenotypes observed.

To further define the extent of the differences between the pex6-1 and pex6-2 alleles, we compared the effects of overexpressing human PEX6 or Arabidopsis PEX5 in these mutants. The pex6-1 mutation alters an Arg residue in the second AAA domain (Figure 6A) that is conserved in human PEX6 (Zolman and Bartel 2004), whereas the Leu residue mutated in pex6-2 is a Met in the human protein and is in a less conserved region (Figure 6A). Expression of a human PEX6 cDNA from the cauliflower mosaic virus 35S promoter (35S:HsPEX6) rescues the IBA resistance and sucrose dependence of pex6-1 (Zolman and Bartel 2004). Similarly, we found that expressing this human PEX6 cDNA restored pex6-1 sensitivity to lateral root promotion by IBA (Figure 7B), rescued pex6-1 PTS2 processing defects (Figure 7C), and partially restored sensitivity of pex6-1 hypocotyls to 2,4-DB in the dark (Figure 7A). In marked contrast, 35S:HsPEX6 did not rescue the strong resistance of pex6-2 lateral roots to IBA (Figure 7B) or the partial resistance of pex6-2 hypocotyls to 2,4-DB (Figure 7A). Our observation that
35S:HsPEX6 failed to rescue the pex6-2 phenotypes assayed suggests that the function(s) disrupted by the pex6-2 mutation is not conserved in the human protein, unlike the pex6-1 mutation (Zolman and Bartel 2004).

pex6-1 exhibits reduced PEX5 levels (Zolman and Bartel 2004), probably because PEX5 is polyubiquitinated and degraded when it is not efficiently removed from the peroxisome by PEX6. PEX5 overexpression from the 35S promoter (35S:PEX5) partially suppresses the sucrose dependence and growth defects of pex6-1 without restoring IBA sensitivity (Zolman and Bartel 2004). In addition, we found that PEX5 overexpression partially restored PTS2 processing in pex6-1 (Figure 7C). Unlike pex6-1, we found normal PEX5 levels in pex6-2 (Figures 2C, 6E, and 7C). In contrast to the beneficial effects of PEX5 overexpression in pex6-1 (Figure 7, A and C), PEX5 overexpression enhanced pex6-2 2,4-DB resistance (Figure 7A) and induced a PTS2 processing defect in pex6-2 (Figure 7C). These enhancements of pex6-2 defects by PEX5 overexpression suggest that unlike pex6-1, pex6-2 defects are not caused by lack of PEX5 available to escort proteins into the peroxisome.

Because the pex6-1 and pex6-2 alleles performed differently in a variety of assays (Figure 6, B-E and Figure 7, A-C), we assessed the ability of each pex6 lesion to complement the defects of the other. F2 plants from a cross of pex6-1 and pex6-2 were assayed for 2,4-DB resistance in roots and individual plants were genotyped. Surprisingly, we found that pex6-1/pex6-2 seedlings were as sensitive to 2,4-DB as wild-type PEX6/PEX6 seedlings (Figure 7D). This intragenic complementation is consistent with our observation that both pex6 alleles accumulated wild-type levels of pex6 protein (Figures 6E and 7C) and implied that the two missense mutations affect separable functions of PEX6.

**Additional pfl mutants**

We used recombination mapping to localize pfl29, pfl99, and pfl106 to distinct chromosome regions (Figure 4). We mapped the pfl29 lesion to the bottom of chromosome 2 using persistent GFP-ICL fluorescence phenotype, pfl99 to the top of chromosome 3 using the
associated phenotype of sucrose dependence, and pfl106 to a region on chromosome 1 using the persistent GFP-ICL fluorescence phenotype (Figure 4). These mapping data indicate that additional loci can mutate to confer GFP-ICL stabilization. Map-based cloning of these additional loci is ongoing.

**Discussion**

*Three classes of pfl mutants based on subcellular GFP-ICL localization*

Although much is known about how matrix proteins enter peroxisomes (reviewed in Hu et al. 2012), little is known about how these matrix proteins are ultimately degraded. The developmentally controlled degradation of the glyoxylate cycle enzymes ICL and MLS provides model substrates with which to unravel peroxisome-associated degradation. We have begun isolating and characterizing mutants with impaired degradation of a GFP-ICL reporter, anticipating that analysis of the defective genes will elucidate the mechanism of peroxisomal matrix protein degradation. We selected mutants that retained GFP-ICL fluorescence longer than wild type, and subcellular GFP-ICL localization has allowed us to separate the mutants into different classes. The first class contains mutants with predominantly cytosolic GFP-ICL, mutants in the second class display both cytosolic and punctate GFP-ICL, and the third class includes mutants with predominantly punctate GFP-ICL (Table 4).

*Import into the peroxisome is needed for efficient ICL degradation*

The pex14-5 and pex14-6 mutants are members of the first class of pfl mutants (Table 4). As illustrated in Figure 8A, PEX14 is a peroxisomal membrane protein (Hayashi et al. 2000) that acts with PEX13 as the PEX5-PEX7 docking complex (Schell-Steven et al. 2005) and may assist PEX5 in forming a transient matrix protein import pore (Meinecke et al. 2010). Whereas pex14-5 resembles previously described pex14 null alleles (Hayashi et al. 2000; Monroe-Augustus et al. 2011), pex14-6 is unique among described Arabidopsis pex14 mutants in displaying sucrose independence (Figure 3B), suggesting that residual pex14-6 protein (Figure 3D) retains some
PEX14 function. The viability of the *pex14*-5 apparent null allele (Figure 3D) confirms a recent report that PEX14, unlike its docking partner PEX13 (Boisson-Dernier et al. 2008), is not required for Arabidopsis viability (Monroe-Augustus et al. 2011). All of the assayed *pex14* alleles similarly stabilize ICL (Figure 3E). ICL and MLS are also stabilized in the *pex5*-10 mutant (Lingard et al. 2009), another peroxin mutant that displays severe matrix protein import defects (Khan and Zolman 2010). These demonstrations that ICL and MLS must enter the peroxisome to be efficiently degraded suggest that either the degradation machinery or the machinery needed to target ICL for destruction is peroxisome-associated (Figure 8B).

**Peroxisomal metabolism can influence ICL degradation**

We found that PED1 promotes efficient peroxisomal matrix protein degradation (Figure 5E). PED1 is a peroxisomal thiolase (Figure 8A) needed for β-oxidation of fatty acids to acetyl-CoA (Hayashi et al. 1998) and of IBA to IAA (Zolman et al. 2000). We were surprised to find that PED1 also was needed for efficient matrix protein import, as judged by both incomplete removal of the PTS2-containing sequence from PMDH (Figure 5E) and partial GFP-ICL mislocalization to the cytosol (Figure 1B) in *ped1* mutants. *ped1* mutants have larger peroxisomes than wild type (Hayashi et al. 1998); perhaps this altered geometry physically impairs matrix protein import. Alternatively, there may exist an undiscovered feedback mechanism linking matrix protein import with peroxisomal metabolism. In either case, ICL stabilization in *ped1* mutants might result from inefficient import of ICL into the peroxisome matrix, as in the *pex14* and *pex5*-10 mutants discussed above. Arguing against this possibility is our observation that *lon2* mutants, which display more severe PTS2 processing defects than *ped1* mutants, fail to stabilize ICL (Figure 5E). An alternative possibility is that reduced β-oxidation in *ped1* lowers peroxisomal H₂O₂, reducing oxidative damage and slowing degradation. Indeed, ICL and MLS are similarly stabilized in a *pxa1* mutant (Lingard et al. 2009) that shows complete sucrose dependence, strong IBA resistance (Zolman et al. 2001), and reduced H₂O₂ levels (Eastmond 2007) due to a reduced ability to move β-oxidation substrates into the peroxisome.
(Figure 8A). Conversely, ICL and MLS degradation is hastened in the cat2 mutant (Lingard et al. 2009), which is missing one of the peroxisomal catalases that decompose H₂O₂. A third non-exclusive possibility is that ICL and MLS degradation may be linked to the depletion of seedling fatty acid stores, which also would explain our observations that ICL degradation is delayed in several mutants with impaired fatty acid β-oxidation. For example, ICL and MLS degradation might be inhibited by fatty acids or β-oxidation intermediates or might be promoted by sucrose or other downstream metabolites of β-oxidation.

The PEX6 ATPase is needed for efficient matrix protein degradation

As illustrated in Figure 8A, PEX4 is a ubiquitin-conjugating enzyme that in yeast and plants is tethered to the peroxisome by PEX22 (Koller et al. 1999; Zolman et al. 2005) and in yeast provides ubiquitin to RING finger peroxins that ubiquitinate the matrix protein receptor PEX5 (Thoms and Erdmann 2006; Platta et al. 2007; Platta et al. 2009). PEX6 and PEX1 are AAA-ATPases that in yeast and mammals assist in the retrotranslocation of ubiquitinated PEX5 from the peroxisome (Figure 8A), thus recycling PEX5 for further import rounds (reviewed in Fujiki et al. 2012; Grimm et al. 2012); PEX5 is poly-ubiquitinated and degraded in the proteasome when PEX6 is not functional (Platta et al. 2007). Arabidopsis PEX6 likely functions similarly to its yeast and mammalian orthologs, as the pex6-1 allele has decreased PEX5 levels and is partially rescued by PEX5 overexpression (Figure 7C and Zolman and Bartel 2004).

By screening for GFP-ICL stabilization, we identified a novel pex6 allele, pex6-2, that shares only a subset of pex6-1 defects, including IBA and 2,4-DB resistance (Figure 6, B-D). Unlike pex6-1, pex6-2 did not require sucrose for normal development in the dark (Figure 6B), processed PTS2 proteins nearly as efficiently as wild type (Figure 6E), and had normal PEX5 levels (Figures 2C, 6E, and 7C). Moreover, pex6-2 physiological and molecular defects were exacerbated rather than rescued by PEX5 overexpression (Figure 7, A and C). Interestingly, the pex6-1 and pex6-2 lesions were able to complement one another (Figure 7D). PEX6 is thought to function as a hexamer (reviewed in Fujiki et al. 2012; Grimm et al. 2012), and this intragenic
complementation suggests that the pex6-1 and pex6-2 missense lesions affect different PEX6 functions, allowing mixed pex6-1 pex6-2 oligomers to carry out all PEX6 functions. Like pex4-l pex22-1 and pex6-1 (Lingard et al. 2009), pex6-2 stabilized ICL and MLS (Figure 6, E and F).

The stabilization of ICL and MLS without dramatic effects on other peroxisomal processes such as matrix protein import (Figure 1E) suggests that ICL and MLS stabilization in pex6-2 does not result from a failure to import ICL and MLS into the peroxisome, as in pex14 alleles. Rather, it seems feasible that peroxisomal matrix proteins require the PEX5-recycling machinery, including PEX6 and PEX4, to move from the peroxisome to the cytosol for proteasomal degradation (Figure 8B).

**Multiple genes contribute to efficient peroxisomal matrix protein degradation**

By screening for mutants exhibiting GFP-ICL stabilization, we have begun identifying genes needed for matrix protein degradation and deciphering the peroxisome-associated matrix protein degradation pathway (Figure 8B). We found that matrix proteins need to enter the peroxisome to be subject to efficient degradation and that the metabolic status of the peroxisome impacts the degradation rate. Moreover, several peroxins involved in ubiquitinating and retrotranslocating PEX5 are needed for efficient degradation, consistent with the intriguing possibility that matrix proteins may leave the peroxisome for proteasomal degradation in the cytosol. The progress reported here also reveals several gaps in our understanding of peroxisome-associated matrix protein degradation that remain to be elucidated, including how matrix proteins are recognized for degradation and how metabolic status is linked to degradation rate. Several pfl mutants for which the defective genes have not been identified displayed neither IBA resistance nor sucrose dependence, but rather appeared to have wild-type β-oxidation phenotypes (Table 4). Identification of the genes defective in these mutants may provide additional insights into how peroxisomal proteins are degraded.
Acknowledgments

We thank John Harada (University of California, Davis) for the MLS antibody, Masayoshi Maeshima (Nagoya University, Japan) for the ICL antibody, Douglas Randall (University of Missouri, Columbia) for the HPR antibody, and Steven Smith and Itsara Pracharoenwattana (University of Western Australia) for the PMDH2 antibody. We are grateful to Wendell Fleming for assistance with confocal microscopy, Daniel Wagner for stereomicroscope use, Lucia Strader and Mauro Rinaldi for recombination mapping markers, and Emily Liljestrand for assistance in mapping lon2-6. We thank the Arabidopsis Biological Resource Center at Ohio State University for seeds from Salk Institute insertion lines, and Lisa Farmer, Wendell Fleming, Kim Gonzalez, Yun-Ting Kao, and Mauro Rinaldi for critical comments on the manuscript. This research was supported by the National Institutes of Health (R01GM079177) and the Robert A. Welch Foundation (C-1309). Confocal microscopy was performed on equipment obtained through a Shared Instrumentation Grant from the National Institutes of Health (S10RR026399-01). M.J.L. was supported in part by a postdoctoral fellowship from the USDA (2008-20659).

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A. Görg, F. Lottspeich and C. Gietl, 2007 Dual specificities of the
Sci. USA 104: 11501-11506.
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Table 2. Primers used for amplification and sequencing of candidate genes

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### Table 3. PCR-based markers for determining genotypes of identified mutations

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¹This is a dCAPS oligonucleotide (Michaels and Amasino 1998; Neff et al. 1998); the underlined nucleotide differs from wild-type sequence to create a restriction site in either the mutant or wild-type PCR amplicon.
Table 4. Classification of *persistent fluorescence* mutants

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<sup>1</sup>In 4-d-old seedlings; C, cytosolic; P, punctate

<sup>2</sup>NT, not tested

<sup>3</sup>In 8-d-old seedlings
Figure Legends

Figure 1. Localization of GFP-ICL using confocal microscopy separates pfl mutants into three categories: (1) cytosolic, (2) both cytosolic and punctate, and (3) punctate patterns. Cotyledon epidermal cells of 4-d-old light-grown Wt (Col-0 transformed with ICLp:GFP-ICL) (A) or pfl mutant (B-I) seedlings were imaged for GFP using confocal microscopy. pfl20/lon2-6 (C), pfl47/pex6-2 (E), and pfl99 (G) display punctate GFP-ICL fluorescence characteristic of peroxisomal localization. Cytosolic GFP-ICL is visible at the cell margins in pfl49/pex14-6 (F) and pfl175/pex14-5 (I). pfl7/ped1-7 (B), pfl29 (D), and pfl106 (H) display both punctate and cytosolic localization. This experiment was repeated twice with similar results. Scale bar = 50 µm.

Figure 2. Most pfl mutants display physiological and/or molecular defects suggestive of peroxisomal defects. (A) Root lengths of 8-d-old pfl or Wt (Col-0) seedlings grown in yellow light in the presence or absence of sucrose or on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (n ≥ 12). (B) Hypocotyl lengths of 6-d-old pfl or Wt (Col-0) seedlings grown in the dark in the presence or absence of sucrose or on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (n ≥ 12). (C) Protein extracts from the 8-d-old seedlings grown in the light on 0.5% sucrose from panel A were processed for immunoblotting. The membrane was serially probed with antibodies to the indicated proteins. The positions of molecular mass markers (in kDa) are indicated at the left. PMDH and thiolase (PED1) are synthesized as precursors (p) containing the PTS2 signal that is processed into the mature (m) protein in the peroxisome. Residual PEX7 (PEX7) from a previous probing remains visible in the PMDH panel. HSC70 is a loading control. Experiments in panels A through C were repeated twice with similar results.
Figure 3. *pex14* mutants display physiological and molecular peroxisomal defects and stabilize ICL. (A) Positions of newly identified *pfl* alleles are shown above and characterized alleles are shown below a *PEX14* gene model in which exons are shown as boxes and introns as lines. (B) Hypocotyl lengths of 7-d-old *pfl* or Wt (Col-0 transformed with *ICLp:GFP-ICL*) seedlings grown in the dark in the presence or absence of sucrose or on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 10). (C) Root lengths of 8-d-old seedlings *pfl* or Wt (Col-0 transformed with *ICLp:GFP-ICL*) grown under yellow-filtered light on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 10). (D) Protein extracts from the 8-d-old seedlings grown in the light on 0.5% sucrose from panel C were processed for immunoblotting. The membrane was serially probed with antibodies to the indicated proteins. The positions of molecular mass markers (in kDa) are indicated at the left. An overexposed anti-PEX14 immunoblot revealed PEX14 protein in all *pex14* alleles except *pex14-5* and *pex14-2*. PMDH is synthesized as a precursor (p) with a cleavable PTS2 signal that is processed into mature (m) PMDH in the peroxisome; this cleavage is impaired in *pex14* mutants. HSC70 is a loading control. (E) ICL is stabilized in *pex14* mutants. Protein extracts from 4-, 6-, and 8-d-old light-grown Wt (Col-0) and *pex14* seedlings were processed for immunoblotting. The membrane was serially probed with antibodies to the indicated proteins. Thiolase is synthesized as a precursor (p) with a cleavable PTS2 signal that is processed into mature (m) thiolase in the peroxisome. HSC70 is a loading control. Experiments in panels B through E were repeated twice with similar results.

Figure 4. Map positions of *pfl* mutants were determined using recombination mapping. Map positions of genes encoding peroxins and selected additional peroxisomal proteins (in black) and bordering mapping markers used (in magenta) are shown to the right of the five *Arabidopsis thaliana* chromosome. Identified *pfl* mutants (in light blue) and unidentified *pfl* mutants (in dark
blue) are shown to the left of the chromosomes with mapping intervals bracketed and the number of recombinants/number of chromosomes scored shown in parentheses (in magenta).

**Figure 5.** Both *lon2* and *ped1* mutants display physiological and molecular peroxisomal defects, but only *ped1* mutants stabilize ICL. (A) Positions of newly identified *pfl* alleles are shown above and characterized alleles are shown below gene models of *LON2* and *PED1*. Green lines above the *LON2* gene model delineate regions encoding the central AAA domain and the C-terminal protease domain. *LON2* and *PED1* encode proteins that are targeted to peroxisomes via a C-terminal PTS1 signal or an N-terminal PTS2 signal, respectively. (B) Hypocotyl lengths of 6-d-old *pfl* or Wt (Col-0) seedlings grown in the dark in the presence or absence of sucrose or on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 12). (C) Root lengths of 8-d-old *pfl* or Wt (Col-0) seedlings grown under yellow-filtered light on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 15). (D) Lateral roots/mm root length of 8-d-old *pfl* or Wt (Col-0) seedlings 4 days after transfer to sucrose-containing medium with or without 10 µM IBA are shown. Error bars show standard deviations of the means (*n* ≥ 8). (E) ICL is stabilized in *ped1* mutants but is degraded similarly to wild type in *lon2* mutants. Protein extracts from 4-, 6-, and 8-d-old light-grown Wt (Col-0) and mutant seedlings were processed for immunoblotting. The membrane was serially probed with antibodies to the indicated proteins. The positions of molecular mass markers (in kDa) are indicated at the left. PMDH and thiolase are synthesized as precursors (p) with a cleavable PTS2 signal that are processed into mature (m) versions in the peroxisome. Residual HPR (HPR) from a previous probing remains visible in the thiolase panel. HSC70 is a loading control. Experiments in panels B, C, and E were repeated twice with similar results.
Figure 6. *pex6*-2 and *pex6*-1 display partially overlapping physiological and molecular peroxisomal defects and stabilize ICL and MLS. (A) The positions of the newly identified *pfl47/pex6*-2 allele and the characterized *pex6*-1 allele are shown above a gene model of PEX6. Green lines above the gene model delineate regions encoding the two PEX6 AAA domains. Arabidopsis PEX6 regions containing the *pex6*-2 and *pex6*-1 lesions are shown below the gene model aligned with orthologs from *Oryza sativa* (NP_001053886), *Selaginella moellendorffii* (XP_002979987), *Mus musculus* (NP_663463), and *Homo sapiens* (NP_000278). (B) Hypocotyl lengths of 6-d-old *pfl* or Wt (Col-0 transformed with *ICLp:GFP-ICL*) seedlings grown in the dark in the presence or absence of sucrose or on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 10). (C) Root lengths of 8-d-old *pfl* or Wt (Col-0 transformed with *ICLp:GFP-ICL*) seedlings grown under yellow-filtered light on sucrose-supplemented medium containing inhibitory concentrations of IBA or 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 8). (D) Lateral roots/mm root length of 8-d-old *pfl* or Wt (Col-0) seedlings 4 days after transfer to sucrose-containing medium with or without 10 µM IBA are shown. Error bars show standard deviations of the means (*n* ≥ 8). (E) Both *pex6* alleles stabilize ICL, whereas only *pex6*-1 displays reduced PEX5 levels or severe PTS2 processing defects. Protein extracts from 4-, 6-, and 8-d-old light-grown Wt (Col-0 transformed with *ICLp:GFP-ICL*) or mutant light-grown seedlings were processed for immunoblotting. Membranes from duplicate gels were serially probed with antibodies to the indicated proteins to obtain the top four panels and the bottom four panels. The positions of molecular mass markers (in kDa) are indicated at the left. PMDH and thiolase are synthesized as precursors (p) with a cleavable PTS2 signal that is processed into mature (m) versions in the peroxisome. An asterisk marks a cross-reacting band detected by the ICL antibody that is not present in an *icl* null mutant (Lingard et al. 2009). HSC70 is a loading control. (F) Both *pex6* alleles stabilize MLS. Protein extracts from 4-, 5-, and 6-d-old Wt (Col-0 transformed with *ICLp:GFP-ICL*) or mutant light-grown seedlings were
processed for immunoblotting with antibodies to MLS and HSC70, a loading control. Experiments in panels B through F were repeated at least twice with similar results.

**Figure 7.** *pex6* complementation analysis. (A) The 2,4-DB resistance of *pex6-1* is fully rescued by the *pBINPEX6* genomic Arabidopsis *PEX6* construct (*PEX6p:* *PEX6*) and partially rescued by expression of a human *PEX6* cDNA (*35S:HsPEX6*) or Arabidopsis *PEX5* overexpression (*35S:PEX5*), whereas *pex6-2* 2,4-DB resistance is rescued by the genomic *PEX6* construct, unaffected by expression of human *PEX6* (two transformants shown), and enhanced by Arabidopsis *PEX5* overexpression (two transformants shown). Hypocotyl lengths of 6-d-old Wt (Col-0) or mutant seedlings grown in the dark on sucrose-supplemented medium containing increasing concentrations of 2,4-DB are shown. Error bars show standard deviations of the means (*n* ≥ 15). (B) The IBA resistance of both *pex6-1* and *pex6-2* lateral root production is fully rescued by a genomic Arabidopsis *PEX6* construct but not by Arabidopsis *PEX5* overexpression. Human *PEX6* expression restores IBA-responsive lateral rooting to *pex6-1* but not to *pex6-2* (two transformants shown). Lateral roots/mm root length of 8-d-old Wt (Col-0) or mutant seedlings 4 days after transfer to sucrose-containing medium with or without 10 µM IBA are shown. Error bars show standard deviations of the means (*n* ≥ 8). (C) The PTS2 processing defect and reduced PEX5 levels of *pex6-1* are rescued by a genomic Arabidopsis *PEX6* construct and by expression of human *PEX6* and are partially rescued by Arabidopsis *PEX5* overexpression; *pex6-2* acquires PTS2 processing defects when Arabidopsis *PEX5* is overexpressed. Protein extracts from the 8-d-old light-grown control seedlings from panel B were processed for immunoblotting. The membrane was serially probed with antibodies to the indicated proteins. The positions of molecular mass markers (in kDa) are indicated at the left. PMDH and thiolase are synthesized as precursors (p) with a cleavable PTS2 signal that are processed into mature (m) proteins in the peroxisome. HSC70 is a loading control. (D) *pex6-1* and *pex6-2* exhibit intragenic complementation of 2,4-DB resistant root elongation. Control and F<sub>2</sub> progeny were plated on media without and with 2,4-DB and root lengths of 8-d-old seedlings
were measured. The genotype of each seedling was then determined. The number of seedlings \( n \) of each genotype is indicated. This intragenic complementation suggests that the \textit{pex6-1} and \textit{pex6-2} missense lesions affect different PEX6 functions, and that mixed oligomers with both \textit{pex6-1} (gray circles) and \textit{pex6-2} (purple circles) can carry out PEX6 (black circles) functions. Experiments in panels A, C, and D were repeated at least twice with similar results.

\textbf{Figure 8.} Arabidopsis peroxisomal matrix protein degradation is influenced by proteins implicated in matrix protein import, receptor recycling, and peroxisomal metabolism. (A) Likely functions of Arabidopsis peroxins (numbered ovals) in peroxisome matrix protein import based on data from Arabidopsis and other systems (reviewed in Hu \textit{et al.} 2012). Matrix proteins are targeted to the peroxisome via a C-terminal PTS1 or an N-terminal PTS2, which are recognized in the cytosol by the PEX5 and PEX7 receptors, respectively. Receptors dock with the membrane peroxins PEX13 and PEX14, deliver cargo, and are recycled. PEX5 recycling requires the ubiquitin-conjugating enzyme PEX4 and a RING-finger complex comprised of PEX2, PEX10, and PEX12. The PEX6 and PEX1 AAA ATPases promote retrotranslocation of ubiquitinated PEX5 out of the peroxisome; in the absence of efficient recycling, PEX5 can be multi-ubiquitinated and degraded in the proteasome. Once in the peroxisome, PTS2 proteins are processed by the peroxisomal protease DEG15 (Helm \textit{et al.} 2007; Schumann \textit{et al.} 2008). Both PTS2 and PTS1 proteins contribute to peroxisome metabolism, including fatty acid and IBA \( \beta \)-oxidation, exemplified by PED1 (Hayashi \textit{et al.} 1998; Zolman \textit{et al.} 2000), the glyoxylate cycle, exemplified by ICL (Eastmond \textit{et al.} 2000a) and MLS (Cornah \textit{et al.} 2004), and \( \text{H}_2\text{O}_2 \) decomposition by catalases including CAT2. PXA1 is a membrane protein that likely transports fatty acids and IBA into the peroxisome (Zolman \textit{et al.} 2001). Mutants defective in proteins shown in color alter the degradation rate of glyoxylate cycle enzymes, including proteins involved in matrix protein import (green), receptor recycling components (blue), and proteins involved in peroxisomal metabolism (brown). (B) A model for peroxisomal matrix protein degradation. Efficient ICL degradation requires PEX5 (Lingard \textit{et al.} 2009) and PEX14 (this
work), implying that ICL import into the peroxisome precedes ICL degradation. Once in the peroxisome, peroxisome metabolism influences the ICL degradation rate, perhaps by modulating the extent of \( \text{H}_2\text{O}_2 \) damage. For example, ICL degradation is slowed in \textit{ped1} (this work) and \textit{pxa1} (Lingard et al. 2009) and is enhanced in a \textit{cat2} mutant (Lingard et al. 2009). The stabilization of ICL (and MLS) in the \textit{pex4-1 pex22-1} mutant (Lingard et al. 2009) and \textit{pex6} mutants (this work) is consistent with the possibility that ICL may exit the peroxisome for cytosolic degradation in the proteasome.
A.

- **Wt**
- **pfl7**
- **pfl20**
- **pfl29**
- **pfl47**
- **pfl49**
- **pfl99**
- **pfl106**
- **pfl164**
- **pfl175**

**Conditions**
- **0.5% sucrose**
- **0.5% sucrose + 10 μM IBA**
- **No sucrose**
- **0.5% sucrose + 2 μM 2,4-DB**

**Y-axis**
- Light-grown root length (mm)

B.

- **Wt**
- **pfl7**
- **pfl20**
- **pfl29**
- **pfl47**
- **pfl49**
- **pfl99**
- **pfl106**
- **pfl164**
- **pfl175**

**Conditions**
- **0.5% sucrose**
- **0.5% sucrose + 30 μM IBA**
- **No sucrose**
- **0.5% sucrose + 3 μM 2,4-DB**

**Y-axis**
- Dark-grown hypocotyl length (mm)

C.

- **PEX5**
- **PEX14**
- **PEX6**
- **PEX7**
- **PMDH (p)**
- **PMDH (m)**
- **thiolase (p)**
- **thiolase (m)**
- **HSC70**
B. Dark-grown hypocotyl length (mm)

- 0.5% sucrose
- 0.5% sucrose + 30 μM IBA
- 0.5% sucrose + 3 μM 2,4-DB

C. Light-grown root length (mm)

- 0.5% sucrose
- 0.5% sucrose + 10 μM IBA
- 0.5% sucrose + 2 μM 2,4-DB

D. Lateral roots per mm root length

- 0.5% sucrose
- 0.5% sucrose + 10 μM IBA

E. Western blot analysis

- lon2-2
- lon2-6
- ped1-96
- ped1-7

Proteins visualized: HSC70, HPR, PMDH (p), PMDH (m)
**A**

![](image1)

**B**

- Dark-grown hypocotyl length (mm)
- Light-grown root length (mm)
- Lateral roots per mm root length

**C**

- 0.5% sucrose
- No sucrose
- 0.5% sucrose + 40 μM IBA
- 0.5% sucrose + 3 μM 2,4-DB

**D**

- 0.5% sucrose
- 0.5% sucrose + 10 μM IBA

**E**

- Wt
- pex6-1
- pex6-2

**F**

- Wt
- pex6-1
- pex6-2

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**A. thaliana** PEX6

Oryza sativa

Selaginella

Mus musculus

Homo sapiens

**ARSARPCVIFFDELDSLAPARGASGDSGGVMDRVVSQLLAEIDGL**

**ARSARPCVIFFDELDSLAPSRGRSGDSGGVMDRVVSQLLAELDGL**

**ARGARPCVIFFDELDALAPARGASGDSGGVMDRVVSQLLAELDGL**

**ARAAAPCIIFFDELDSLAPSRGRSGDSGGVMDRVVSQLLAELDGL**

**H. sapiens**

- A. thaliana PEX6
- Oryza sativa PEX6
- Selaginella PEX6
- Mus musculus PEX6
- Homo sapiens PEX6
IBA (2,4-DB) and IAA (2,4-D) affect fatty acid acetyl-CoA.

PTS1 cargo participates in growth, root elongation inhibition, and lateral root development.

CAT2 catalyzes the conversion of H2O2 to H2O and O2, protecting against H2O2 damage or other signal(s).

PTS2 cargo involves peroxisomal metabolism and β-oxidation, with receptor poly-ubiquitination and proteasomal degradation.

H2O2 damage or other signal(s) triggers receptor de-ubiquitination and recycling, as well as matrix protein poly-ubiquitination and proteasomal degradation.