

# *pex5* Mutants That Differentially Disrupt PTS1 and PTS2 Peroxisomal Matrix Protein Import in Arabidopsis<sup>1[W][OA]</sup>

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PEX5 and PEX7 are receptors required for the import of peroxisome-bound proteins containing one of two peroxisomal targeting signals (PTS1 or PTS2). To better understand the role of PEX5 in plant peroxisomal import, we characterized the Arabidopsis (*Arabidopsis thaliana*) *pex5-10* mutant, which has a T-DNA insertion in exon 5 of the *PEX5* gene. Sequencing results revealed that exon 5, along with the T-DNA, is removed in this mutant, resulting in a truncated *pex5* protein. The *pex5-10* mutant has germination defects and is completely dependent on exogenous Suc for early seedling establishment, based on poor utilization of seed-storage fatty acids. This mutant also has delayed development and reduced fertility, although adult *pex5-10* plants appear normal. Peroxisomal metabolism of indole-3-butyric acid, propionate, and isobutyrate also is disrupted. The *pex5-10* mutant has reduced import of both PTS1 and PTS2 proteins, and enzymatic processes that occur in peroxisomes are disrupted. To specifically study the import and importance of PTS1 proteins, we made a truncated *PEX5* construct lacking the PTS1-binding region (*PEX5*<sub>454</sub>). Transformation of this construct into *pex5-10* resulted in the rescue of PTS2 import, thereby creating a line with PTS1-specific import defects. The *pex5-10* (*PEX5*<sub>454</sub>) plants still had developmental defects, although restoring PTS2 import resulted in a less severe mutant phenotype. Comparison of *pex5-10* and *pex5-10* (*PEX5*<sub>454</sub>) phenotypes can separate the import mechanisms for enzymes acting in different peroxisomal processes, including indole-3-butyric acid/2,4-dichlorophenoxybutyric acid oxidation, isobutyrate and propionate metabolism, and photorespiration.

Peroxisomes house a number of critical processes in plants (Kaur et al., 2009). They are the primary site for fatty acid  $\beta$ -oxidation and play an essential role during germination and early seedling establishment in oil-seed plants. Prior to photosynthetic initiation, stored lipids are  $\beta$ -oxidized in peroxisomes to provide the necessary energy and metabolites for development. Peroxisomes also play a role in plant hormone  $\beta$ -oxidation. They are the site for the conversion of an auxin storage form, indole-3-butyric acid (IBA), to its active signaling counterpart, indole-3-acetic acid (IAA; Zolman et al., 2000; Strader et al., 2010). The latter reactions of the jasmonic acid (JA) synthesis pathway also take place in peroxisomes (Wasternack and Kombrink, 2010). Metabolism of intermediates such as propionate, isobutyrate, acetate, and benzoic acid occurs in peroxisomes (Zolman et al., 2001; Hooks et al., 2007; Lucas et al., 2007; Ibdah and Pichersky, 2009). As a result of these metabolic processes, peroxisomes affect important physiological processes, including senescence, starvation, photorespiration, and photomor-

phogenesis (Wanner et al., 1991; Graham et al., 1992; Hu et al., 2002; Foyer et al., 2009; Kunz et al., 2009).

Since peroxisomes lack genetic material, proteins acting in peroxisomal processes are synthesized on free ribosomes in the cytosol and imported posttranslationally. These proteins contain one of two well-characterized peroxisomal targeting signals (PTS1 and PTS2) that direct target proteins to the peroxisome matrix. PTS1 is a C-terminal tripeptide consisting of the consensus sequence (S/A/C)(K/R/H)(L/M); in plants, the preferred targeting signal is Ser-Lys-Leu (SKL; Reumann et al., 2007). This signal is not removed after import. PTS2 is at the N terminus of target proteins and consists of nine amino acids; the most common plant PTS2 is RL-X<sub>5</sub>-HL (Reumann et al., 2004). The N-terminal signal sequence is cleaved after import. Approximately 400 genes in the Arabidopsis (*Arabidopsis thaliana*) genome code for proteins with either PTS1 or PTS2 signals (Kamada et al., 2003; Reumann, 2004; Reumann et al., 2007; Kaur et al., 2009). A majority of these proteins contain a PTS1, with about one-quarter having the PTS2 sequence (Kamada et al., 2003; Reumann, 2004; Reumann et al., 2007).

Proteins that play a role in peroxisome biogenesis are referred to as PEROXINS (PEX). PEX5 and PEX7 function as receptors that recognize PTS1 and PTS2 proteins, respectively, for matrix protein import. PTS1 protein import occurs when the PTS1-containing protein is bound by PEX5 in the cytoplasm. The receptor-cargo complex interacts with the peroxisome membrane proteins PEX13 and PEX14 (Hayashi et al., 2000; Nito et al., 2002; Mano et al., 2006). The complex then translocates the matrix protein into the peroxisome in a process that

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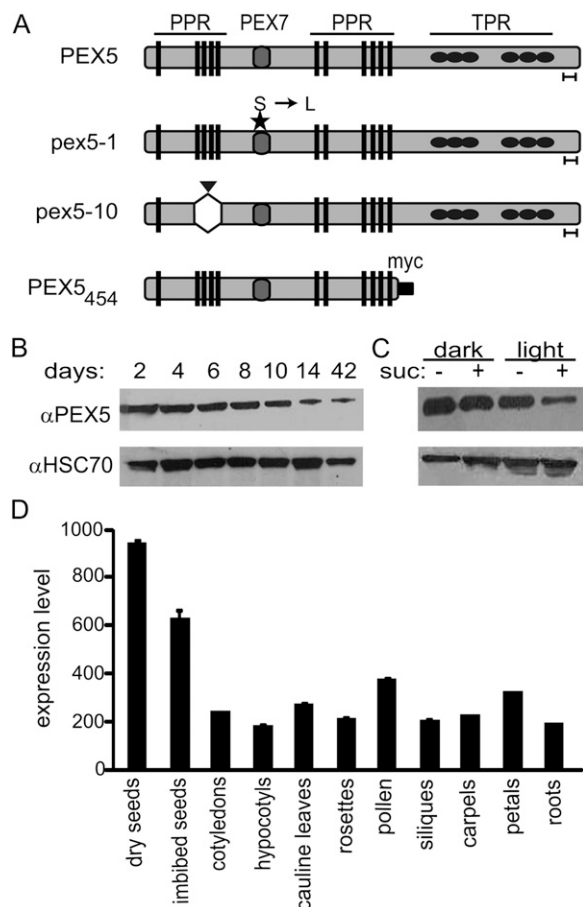
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requires the RING-finger proteins PEX2, PEX10, and PEX12 (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005). After translocation into the matrix, the cargo is released and the receptor is recycled to the cytosol for additional rounds of import (Dodt and Gould, 1996; Dammai and Subramani, 2001; Nair et al., 2004). Receptor recycling requires the PEX1 and PEX6 ATPases and the PEX4 ubiquitin-conjugating enzyme, which is anchored to the peroxisome membrane by PEX22 (Geisbrecht et al., 1998; Kiel et al., 1999; Zolman and Bartel, 2004; Zolman et al., 2005). In yeast, monoubiquitination of PEX5 by PEX12 targets the receptor for recycling back to the cytosol, allowing additional rounds of import (Platta et al., 2009). PTS2-containing proteins are imported into peroxisomes by the PEX7 receptor. PEX7-mediated import requires PEX5 for peroxisomal membrane protein interactions (Nito et al., 2002; Hayashi et al., 2005; Woodward and Bartel, 2005). Recent evidence also indicates that PEX5 requires PEX7 for optimal peroxisomal import in Arabidopsis (Ramón and Bartel, 2010).

Arabidopsis PEX5 contains three regions critical for its function: the tetratricopeptide repeat (TPR) region, the pentapeptide repeat (PPR) region, and a PEX7-binding domain (Fig. 1A). In its C terminus, PEX5 has six TPRs, which are 34-amino acid conserved repeats. The TPR interacts with the PTS1 sequence of proteins destined for import (Dodt et al., 1995; Terlecky et al., 1995; Gatto et al., 2000). In the N terminus, PEX5 contains 11 PPRs, which interact with peroxisomal membrane proteins during translocation (Nito et al., 2002). This region also affects the recycling of PEX5 back to the cytosol (Costa-Rodrigues et al., 2004). PEX5 has a central PEX7-binding domain (Nito et al., 2002), allowing it to bind the PTS2 receptor.

Genetic analysis has indicated that numerous PEX proteins are required for correct peroxisomal matrix protein import in Arabidopsis. Strong loss-of-function mutations resulted in embryo-lethal mutants, as seen in *pex2* and *pex10* (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003). However, partial loss-of-function mutants have revealed characteristic phenotypes associated with decreased peroxisome function. Peroxisome-defective mutants are unable to efficiently metabolize long-chain fatty acids. This deficiency can be directly measured by monitoring the metabolism of C20:1 (eicosenoic acid), which is high in seeds but quickly metabolized (Lemieux et al., 1990). Alternatively, because mutants break down seed-storage fatty acid substrates more slowly, providing less energy, the plants show delayed development and a "Suc-dependent" phenotype: mutants are unable to establish into adults, unless provided exogenous Suc. Other processes commonly are disrupted in peroxisome-defective mutants, including the conversion of IBA to IAA (Zolman et al., 2000). This deficiency can be seen in a root elongation assay; peroxisome-defective mutants are resistant to the inhibitory effects of exogenous hormone.

Because PEX5 plays a critical role in peroxisomal matrix protein import, we further investigated the



**Figure 1.** PEX5 gene expression and protein concentration. A, Cartoon representation of the PEX5 protein, the *pex5* mutant proteins, and the PEX5<sub>454</sub> truncation. The *pex5-1* Ser-to-Leu point mutation (Zolman et al., 2000) and the *pex5-10* insertion site and resulting deletion are indicated by a star and a triangle, respectively. Gray circles indicate the PEX7-binding domain (PEX7), black ovals indicate the TPR domains, and rectangles represent the PPR domains. The goalpost under the protein indicates the recognition site of the PEX5 antibody (Zolman and Bartel, 2004). B, Total protein was extracted from wild-type Col-0 seedlings and adult plants and analyzed by western blot for PEX5 levels. C, Total protein from 5-d-old wild-type seedlings grown in the dark or light in the presence or absence of Suc was extracted and analyzed by western blot for PEX5 levels. For both B and C, equal amounts of protein were loaded, as confirmed by immunoblotting with an HSC70 antibody (bottom). D, Levels of PEX5 mRNA expression (absolute values) in dry and imbibed seeds, cotyledons, hypocotyls, cauline leaves, rosette leaves, mature pollen, seeds/siliques, carpels, petals, and roots. The graph was constructed using Arabidopsis eFP Browser data from <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi> (Winter et al., 2007). Data were retrieved on March 6, 2010.

activity of this protein in Arabidopsis. Previously, the *pex5-1* mutant was identified as an IBA-resistant mutant with weak Suc dependence and a slight decrease in seed-storage fatty acid metabolism (Zolman et al., 2000). *pex5-1* has a C-to-T point mutation, causing a Ser-to-Leu amino acid conversion in the region of PEX5 responsible for binding PEX7 (Zolman et al., 2000). The *pex5-1* mutant has a PTS2 protein import

defect, indicated by reduced PTS2-GFP peroxisomal localization and normal GFP-PTS1 import (Woodward and Bartel, 2005). In this work, we characterized phenotypic abnormalities in the *pex5-10* insertion mutant. *pex5-10* exhibits a more severe phenotype than *pex5-1*, including disruption of both PTS1 and PTS2 import. However, we show that the insertion mutant is not a null allele and likely survives because of an unusual T-DNA processing that allows the production of a partial transcript. Finally, we created a PTS1-specific *pex5* line by complementing *pex5-10* with a partial *PEX5* cDNA, restoring PTS2 import. Comparison of *pex5-10* with mutant transgenic lines expressing the truncated *PEX5*<sub>454</sub> protein allowed us to separate the effects of defects in PTS1, PTS2, or PTS1 and PTS2 import.

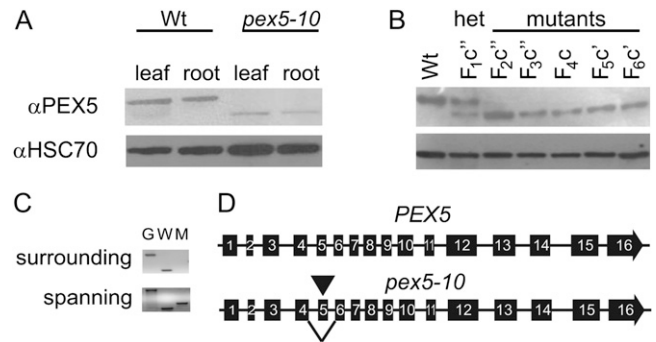
## RESULTS

### PEX5 Levels Are Highest Early in Development

We began our study of *PEX5* by investigating how the protein accumulates in plants. Western blots using an antibody that recognizes the C terminus of *PEX5* (Zolman and Bartel, 2004) revealed that the protein accumulated at high levels in seedlings and at lower levels as plants mature (Fig. 1B). Because peroxisomal metabolism, and hence *PEX5*, are critical during early seedling development when fatty acids are being highly metabolized, we also checked the accumulation of the protein under different developmental conditions. *PEX5* was present at a higher level in the dark than in the light (Fig. 1C). In addition, we saw higher accumulation in the absence of Suc than when Suc was included in the medium (Fig. 1C). These protein patterns are consistent with  $\beta$ -oxidation increases during dark development, when photosynthesis cannot occur, and with decreases when Suc is present, indicating that photosynthesis is high. These protein studies are consistent with publicly available microarray data, which show that *PEX5* gene expression is highest in seeds but relatively stable throughout plant development (Fig. 1D).

### *pex5-10* Encodes an Altered *pex5* Protein

To study the role of *PEX5* in plants and examine the effects of peroxisomal import during plant development, we wanted to examine a stronger mutant allele than the previously described *pex5-1* point mutation (Zolman et al., 2000). The *pex5-10* mutant has a T-DNA insertion in exon 5 (Fig. 1A), which we confirmed by PCR amplification using genomic and T-DNA-specific primers (data not shown). Sequence analysis of the mutant line revealed that the site of the insertion was at position 1,769 (with 1 being the A of the ATG initiator). Using western blots with a *PEX5* antibody, we detected no full-length protein in the mutant (Fig. 2A). However, we saw a lower molecular weight product (Fig. 2A), which segregated in an F1 backcross plant (Fig. 2B). The smaller protein product typically



**Figure 2.** T-DNA insertion effects in the *pex5-10* mutant. A, Total protein extracted from leaves and roots of 12-d-old wild-type (Wt) and *pex5-10* seedlings was analyzed by western blot with a *PEX5* antibody. B, Total protein from 3-week-old wild-type and *pex5-10* plants was analyzed by western blot for *PEX5* levels. Seedlings were taken from the heterozygous progeny of a *pex5-10* backcross to Col-0 ( $F_1c'$ ) and *pex5-10* homozygous mutant plants over four generations ( $F_2$  through  $F_6$ ) and three backcrosses ( $F_{4c}$  for the first backcross;  $F_{5c'}$  and  $F_{6c'}$  for the second backcross;  $F_{2c''}$  and  $F_{3c''}$  for the third backcross). For all blots, equal amounts of protein were loaded, as confirmed by immunoblotting with an HSC70 antibody (bottom). C, To examine the effects of the insertion in exon 5, cDNAs from 3-week-old wild-type (W) and *pex5-10* (M) plants were amplified with *PEX5* primers that directly surround (exons 5 + 6; expected cDNA size 340 bp/expected genomic size 774 bp) or span (exons 3 + 7; 780 bp/2,234 bp) the T-DNA. Genomic DNA (G) from wild-type plants was amplified to show the purity of cDNA synthesis. D, Cartoon representation of the *PEX5* and *pex5-10* genomic structures. Exon numbers are labeled. The *pex5-10* insertion is shown by the triangle above the sequence, and the splice transition from the end of exon 4 to the beginning of exon 6 is indicated.

was present at a lower abundance but was detectable consistently through several generations and following additional backcrosses to the wild type (Fig. 2B).

To determine the mutant protein sequence, we isolated cDNA from mutant plants and performed reverse transcription (RT)-PCR using *PEX5*-specific primers. We did not see a product using primers directly surrounding the insertion, consistent with our PCR data. However, we could see products amplified using either upstream or downstream primer sets (data not shown). Interestingly, we observed smaller than expected products when we used spanning primers around the insertion (Fig. 2C). We sequenced the spanning product and found a complete deletion of exon 5 along the normal exon/intron junctions in the mutant cDNA, with the insertion removed (Fig. 2D). This excision resulted in the production of a mutant protein lacking four of the PPRs (Fig. 1A). The abnormal protein accumulated in both leaves and roots of adult plants at similar levels, indicating that this alteration was not tissue specific (Fig. 2A).

### *pex5-10* Has Defects in Both PTS1 and PTS2 Import

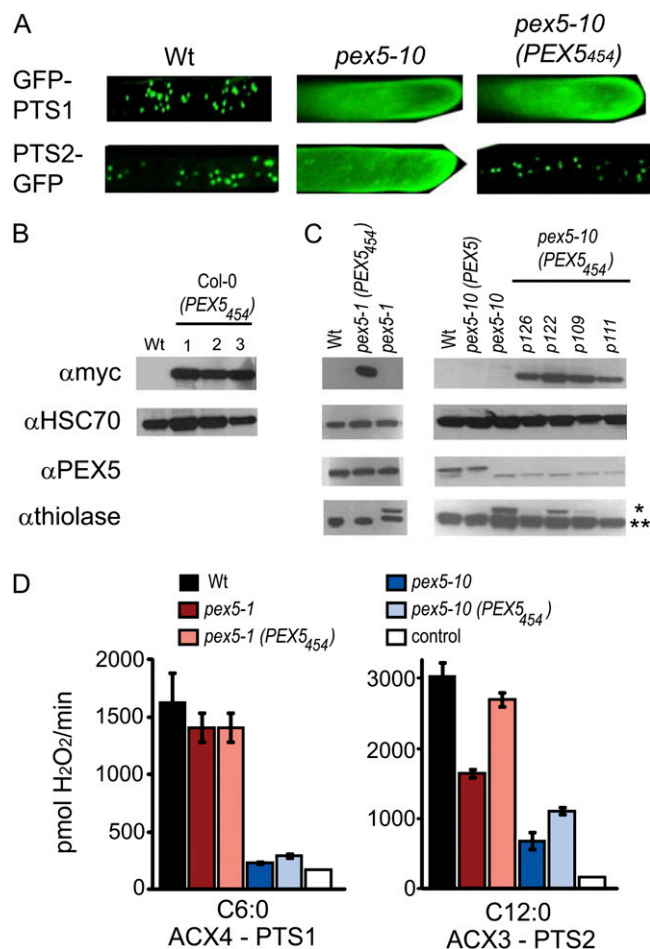
One rationale for studying *pex5* mutants was to determine the effects of losing PTS1 import into peroxisomes. However, the *pex5-1* mutant has a PTS2-specific defect (Woodward and Bartel, 2005). To demonstrate

the import defects in *pex5-10*, we crossed the mutant with lines containing GFP reporter genes targeted to peroxisomes using either the PTS1 (GFP-PTS1; Zolman and Bartel, 2004) or PTS2 (PTS2-GFP; Woodward and Bartel, 2005) sequence. Peroxisomal import in wild-type plants appears as a characteristic punctate patterning. Compared with the wild-type controls, *pex5-10* showed a diffuse pattern indicative of cytosolic mislocalization for both PTS1- and PTS2-containing proteins (Fig. 3A). This result demonstrates that both peroxisomal import pathways are disrupted, consistent with previous studies showing disruptions in the import of PTS1 proteins (Lee et al., 2006; Lingard and Bartel, 2009; Lingard et al., 2009) and PTS2 proteins (Zolman et al., 2005; Lee et al., 2006; Lingard and Bartel, 2009; Ramón and Bartel, 2010).

### Overexpression of a Truncated PEX5 Rescues PTS2 Import Defects

To further investigate the role of PEX5, we wanted to create a *pex5* mutant plant line with a PTS1-specific import defect. We designed six constructs to express truncated PEX5 proteins using the cauliflower mosaic virus 35S promoter. Each of these constructs was independently transformed into wild-type ecotype Columbia (Col-0) plants. However, five of the mutant proteins did not accumulate in wild-type plants selected to contain the transgene (data not shown). The *PEX5*<sub>454</sub> construct encodes the first 454 amino acids of the protein, including the PPRs important for peroxisomal membrane protein binding and the PEX7 interaction domain; this construct does not have any of the C-terminal TPR domains known to bind the PTS1 of peroxisome-bound proteins (Fig. 1A). Because our PEX5 antibody recognizes the C terminus of the full-length protein, we added a myc epitope tag to *PEX5*<sub>454</sub>. We analyzed wild-type Arabidopsis transformants for mutant protein accumulation using  $\alpha$ myc (Fig. 3B). Col-0 (*PEX5*<sub>454</sub>) plants with a strong  $\alpha$ myc signal then were crossed to *pex5-1* and *pex5-10* mutants. We selected mutant lines containing the transgene by PCR, then confirmed the accumulation of the *PEX5*<sub>454</sub> protein (Fig. 3C). Because we saw some variability in *pex5-10* (*PEX5*<sub>454</sub>) responses, four independent lines (designated px) are shown for most assays to represent the range of phenotypes. As a control for phenotypic experiments, we used *pex5-10* complemented with a full-length *PEX5* cDNA.

Our hypothesis was that *PEX5*<sub>454</sub> would restore PTS2 import in the *pex5* mutants, given that the *PEX5*<sub>454</sub> protein retains the regions necessary for PEX7 binding and peroxisomal membrane interactions. To determine the effects of the transgene on PTS2 import, we examined the import of the GFP reporter genes and compared the responses with the mutant parents. We saw restored PTS2-GFP import in *pex5-10* (*PEX5*<sub>454</sub>) compared with the *pex5-10* parent line (Fig. 3A). As hypothesized, PTS1 protein import was similarly impaired in the transgenic lines and the *pex5-10* parent line (Fig. 3A). The



**Figure 3.** Peroxisomal import defects in *pex5* mutant lines. A, Localization of peroxisomally targeted GFP reporters with either PTS1 (Zolman and Bartel, 2004) or PTS2 (Woodward and Bartel, 2005) sequence. Wild-type (Wt) lines (Col-0 background) containing these constructs were crossed into the *pex5-10* or *pex5-10* (*PEX5*<sub>454</sub>) background. Root hair cells for each line are shown, with all images taken at the same magnification. B, Total protein from wild-type and three independent transgenic Col-0 (*PEX5*<sub>454</sub>) plants were analyzed by western blot for *PEX5*<sub>454</sub> accumulation using a myc antibody. C, Western blot of total protein from wild-type, *pex5-10* (*PEX5*), *pex5-1*, *pex5-1* (*PEX5*<sub>454</sub>), *pex5-10*, and *pex5-10* (*PEX5*<sub>454</sub>) with *PEX5*, myc, and thiolase antibodies. Thiolase contains a PTS2 that is cleaved following import; protein size indicates if the protein is localized in the peroxisome (mature short form; two asterisks) or if it remains in the cytoplasm (unprocessed long form; asterisk). Four independent *pex5-10* (*PEX5*<sub>454</sub>) lines (p126, p122, p109, and p111) are shown to indicate the range of responses. For all blots, equal amounts of protein were loaded, as confirmed by immunoblotting with an HSC70 antibody. D, Extracts from 4-d-old Col-0 (wild-type), *pex5-1*, *pex5-1* (*PEX5*<sub>454</sub>), *pex5-10*, and *pex5-10* (*PEX5*<sub>454</sub>) seedlings were tested for acyl-CoA oxidase activity on *n*-hexanoyl-CoA (C6:0) and lauroyl-CoA (C12:0). Data are presented as pmol H<sub>2</sub>O<sub>2</sub> produced per min. Error bars represent  $\pm$  SE of the rates from three independent experiments. The control samples used water in place of plant extracts to account for background readings.

*pex5-1* (*PEX5*<sub>454</sub>) line showed a similar recovery of PTS2 import compared with the *pex5-1* control (data not shown).

We further examined the import and processing of the PTS2 protein 3-ketoacyl-CoA thiolase, which acts in the final step of  $\beta$ -oxidation (Hayashi et al., 1998). PTS2 proteins are synthesized in the cytosol, and the N-terminal signal sequence is proteolytically cleaved by the DEG15 protease following import into the peroxisomal matrix, resulting in a smaller mature protein (Hijikata et al., 1987; Helm et al., 2007; Schuhmann et al., 2008). Analysis of thiolase processing from the larger cytosolic form to the smaller mature form by western blot can serve as one indicator of the degree of peroxisomal import. Both *pex5-1* and *pex5-10* showed defects in thiolase processing, with *pex5-10* being more severe (Fig. 3C). However, inclusion of the *PEX5<sub>454</sub>* transgene in *pex5-1* mutants led to a complete rescue of thiolase processing. The *pex5-10* (*PEX5<sub>454</sub>*) lines still showed defects in thiolase processing to varying degrees, but each showed improvement compared with the parent phenotype (Fig. 3C). The remaining processing defect in *pex5-10* (*PEX5<sub>454</sub>*) may be due to decreased import of DEG15, which uses a PTS1, as we did not see residual defects in *pex5-1* (*PEX5<sub>454</sub>*) lines or *pex5-10* (*PEX5*) lines, in which PTS1 import was normal. These results further suggest that the *PEX5<sub>454</sub>* protein can improve PTS2 import in both *pex5-1* and *pex5-10* mutant plants.

To characterize the consequences of the import defects on peroxisomal function, we measured the activity of two acyl-CoA oxidase (ACX) enzymes, ACX3 and ACX4. These enzymes act in the first step of  $\beta$ -oxidation (Graham, 2008). ACX3 has a PTS2 and ACX4 has a PTS1. Activity assays indicate that ACX3 primarily  $\beta$ -oxidizes medium-chain fatty acids, such as C12:0 (Eastmond et al., 2000; Froman et al., 2000; Adham et al., 2005) and that ACX4  $\beta$ -oxidizes short-chain substrates of C4:0 to C6:0 (Hayashi et al., 1999; Rylott et al., 2003; Adham et al., 2005). Extracts prepared from *pex5-1* had similar activity as the wild type on C6:0 substrates, but *pex5-10* showed significantly reduced activity on this substrate (Fig. 3D). These data confirm that PTS1 protein activity is not disrupted in *pex5-1* but is severely compromised in *pex5-10*. The *pex5-1* and *pex5-10* mutants were both impaired in  $\beta$ -oxidation of C12:0 fatty acid substrates, consistent with the defects that these mutants have in PTS2 import (Fig. 3D). Addition of *PEX5<sub>454</sub>* to *pex5-1* rescued C12:0 activity to wild-type levels and partially rescued the *pex5-10* mutant (Fig. 3D). These results suggest that the *pex5* PTS2 import defects are reduced by inclusion of the *PEX5<sub>454</sub>* protein. The activity assays showed similar C6:0 fatty acid defects in *pex5-10* and *pex5-10* (*PEX5<sub>454</sub>*) (Fig. 3D). This result further suggests that the *pex5-10* PTS1 protein import defect is not improved by the *PEX5<sub>454</sub>* protein.

#### *pex5-10* Mutants Have Severe Defects in $\beta$ -Oxidation

The most striking phenotype for the *pex5-10* mutant was a severe germination defect. Typical germination rates for *pex5-10* lines ranged from 0% to 20%, which were seed line and harvest date dependent (data not

shown). Any germination that occurred was delayed compared with the wild type; by day 3, 100% of wild-type and *pex5-1* mutant seeds had germinated but none of the *pex5-10* seeds had germinated (Table I). To quantify germination rates, we plated progeny seeds from an F2 segregating population of a *pex5-10*  $\times$  Col-0 backcross. After 4 d, approximately 75% of the seeds had germinated, but we never identified *pex5-10* mutant seedlings (Table II). We saw the expected number of heterozygous plants (Table II), indicating that the mutation segregated in a recessive fashion. Interestingly, if we nicked the seed coats of seeds that did not germinate, we saw increased germination, as defined by radicle emergence. Genotyping results indicated that this population represented the homozygous mutant lines (Table II).

Although *pex5-10* seeds germinated following nicking, hypocotyl elongation still did not occur on medium lacking Suc (Fig. 4A). However, nicked *pex5* seedling hypocotyls could elongate on Suc-supplemented medium in a concentration-dependent manner (Fig. 4A). This phenotype is consistent with a peroxisome-defective phenotype, as loss of fatty acid  $\beta$ -oxidation coincides with an inability to develop that can be rescued by the application of Suc (Hayashi et al., 1998). This phenotype is similar to, but more severe than, the *pex5-1* phenotype (Fig. 4A).

To determine whether the *PEX5<sub>454</sub>* protein rescued the germination defects in *pex5-1* and *pex5-10*, we assayed the transgenic lines for reduced Suc dependence. The results showed that the *PEX5<sub>454</sub>* protein restored *pex5-1* growth without Suc (Fig. 4B). Although *pex5-10* seeds did not germinate without nicking, seeds of *pex5-10* (*PEX5<sub>454</sub>*) lines could germinate (Table I) and hypocotyls could elongate in medium supplemented with Suc (Fig. 4B). When seed coats were nicked, the *pex5-10* (*PEX5<sub>454</sub>*) lines had enhanced hypocotyl elongation compared with *pex5-10*, although still reduced compared with the wild type (Fig. 4C). Additionally, the *pex5-10* (*PEX5<sub>454</sub>*) seedlings established more robustly than the *pex5-10* seedlings (Fig. 4D).

To directly examine fatty acid  $\beta$ -oxidation, we quantified levels of the seed-storage fatty acid eicosenoic acid in 6-d-old seedlings. Eicosenoic acid is high in seeds but quickly is degraded (Lemieux et al., 1990); wild-type seedlings show only approximately 4%

**Table I.** Unassisted germination rate of 100 seeds over time

Data are shown as percentage germination.

| Seed Lines                                        | Day 2 | Day 3 | Day 4 |
|---------------------------------------------------|-------|-------|-------|
| Wild type                                         | 7     | 100   | 100   |
| <i>pex5-10</i>                                    | 0     | 0     | 1     |
| <i>pex5-10</i> ( <i>PEX5</i> )                    | 9     | 100   | 100   |
| <i>pex5-1</i>                                     | 10    | 100   | 100   |
| <i>pex5-1</i> ( <i>PEX5<sub>454</sub></i> )       | 74    | 100   | 100   |
| <i>pex5-10</i> ( <i>PEX5<sub>454</sub></i> ) p111 | 0     | 38    | 100   |
| <i>pex5-10</i> ( <i>PEX5<sub>454</sub></i> ) p126 | 1     | 47    | 99    |
| <i>pex5-10</i> ( <i>PEX5<sub>454</sub></i> ) p109 | 8     | 50    | 100   |
| <i>pex5-10</i> ( <i>PEX5<sub>454</sub></i> ) p122 | 3     | 33    | 100   |

**Table II.** Germination responses of 100 segregating seeds from four progeny of a *pex5-10* × *Col-0* backcross

Data are shown as number of individuals.

| Genotype       | Parent 1 | Parent 2 | Parent 3 | Parent 4 |
|----------------|----------|----------|----------|----------|
| Wild type      | 19       | 21       | 26       | 28       |
| Heterozygous   | 52       | 54       | 52       | 49       |
| <i>pex5-10</i> | 0        | 0        | 0        | 0        |
| After nicking  |          |          |          |          |
| Wild type      | 0        | 0        | 0        | 0        |
| Heterozygous   | 0        | 0        | 0        | 0        |
| <i>pex5-10</i> | 24       | 25       | 20       | 23       |

eicosenoic acid after 6 d (Fig. 4E). However, eicosenoic levels remained at approximately 25% in *pex5-10* seedlings and at 10% in *pex5-1* (Fig. 4E). These results indicate that the *pex5* mutants have reduced  $\beta$ -oxidation of seed-storage fatty acids, with phenotypic strength paralleling the genetic defects. In contrast to *pex5-1*, *pex5-1* (*PEX5<sub>454</sub>*) levels were similar to wild-type levels (Fig. 4E). *pex5-10* (*PEX5<sub>454</sub>*) lines showed improvements in metabolism from 25% eicosenoic acid to 23% to 17%, indicative of a somewhat increased  $\beta$ -oxidation rate in the transgenic lines compared with the mutant parent (Fig. 4E).

Taken together, these results indicate that the *pex5* mutants have disruptions in peroxisomal function that can be visualized by developmental defects. The *PEX5<sub>454</sub>* protein restored *pex5-1* hypocotyl elongation and fatty acid  $\beta$ -oxidation back to wild-type levels. *pex5-1* has a PTS2-specific defect, and the complete rescue of this phenotype in the transgenic lines is consistent with the *PEX5<sub>454</sub>* protein increasing PTS2 import. The truncated protein only partially rescued the severe fatty acid  $\beta$ -oxidation defects in the *pex5-10* mutant, which has both PTS1 and PTS2 import defects. Although this rescue is only partial, the *PEX5<sub>454</sub>* addition does provide sufficient energy for the *pex5-10* (*PEX5<sub>454</sub>*) seeds to germinate without physical assistance.

#### *pex5-10* Mutants Have Reduced Responses in Peroxisomal Hormone Metabolism

To investigate the extent of peroxisomal defects in the *pex5* mutants, we next examined the responses of seedlings to IBA and 2,4-dichlorophenoxybutyric acid (2,4-DB), a synthetic IBA analog (Figs. 5 and 6). These compounds are metabolized in peroxisomes. Both *pex5-1* and *pex5-10* seedlings were insensitive to the inhibitory effects of IBA and 2,4-DB on root elongation (Fig. 5). *pex5-10* mutants have short roots on unsupplemented medium, but root length actually increased on 5 to 10  $\mu$ M IBA and remained longer than the wild type over a range of IBA concentrations (Fig. 5B). Expression of *PEX5<sub>454</sub>* restored IBA sensitivity to *pex5-1* mutants (Fig. 5A). However, this truncated *PEX5* protein only rescued the IBA insensitivity of *pex5-10* partially (Fig. 5C), and the degree of rescue varied among lines. In contrast, the *pex5-1* (*PEX5<sub>454</sub>*) and *pex5-10* (*PEX5<sub>454</sub>*) lines were all similarly sensitive to 2,4-DB (Fig. 5E).

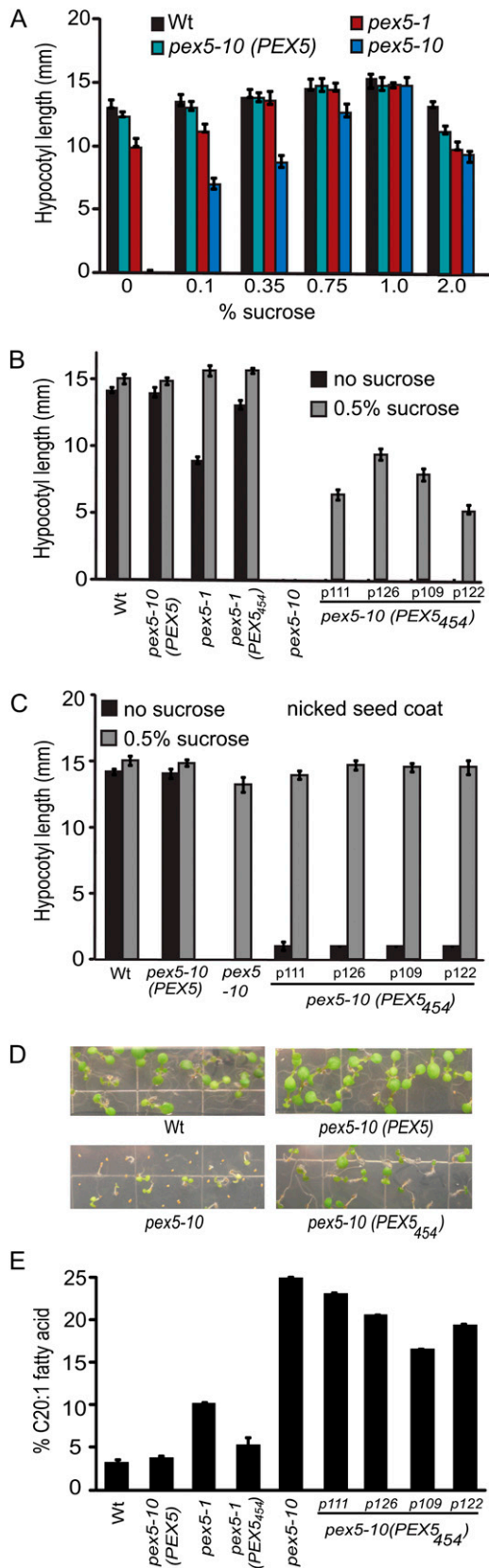
The *pex5* mutants and the *PEX5<sub>454</sub>* lines responded normally to the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthylacetic acid (NAA; Fig. 7). However, as seen with IBA and 2,4-DB, *pex5-10* often showed increased growth on low concentrations of IAA (Fig. 7A), perhaps indicating that IAA levels are low in the mutant or that exogenous application increases the ability of the mutant to develop.

Because one role of IBA in plants is the initiation of lateral roots, we examined mutants for lateral root formation. Both *pex5* mutants were compromised in lateral root formation on medium without hormone or with IBA (Fig. 6). On medium supplemented with IBA, the *PEX5<sub>454</sub>* protein rescued *pex5-1* seedling defects to wild-type levels, but lateral root formation was again only partially rescued in *pex5-10* (*PEX5<sub>454</sub>*) seedlings (Fig. 6A). In contrast, *pex5* mutants initiated lateral roots similar to the wild type on NAA (Fig. 6A), indicating that the defect is not in lateral root development but in lateral root stimulation. This defect can be visualized in *pex5-10* (*DR5-GUS*) lines, which contain an auxin-inducible reporter (Guilfoyle, 1999) that highlights lateral root primordia. *pex5-10* lines with this transgene show reduced GUS levels and fewer lateral roots than *Col-0* (*DR5-GUS*) lines (Fig. 6B). Taken together, these data suggest that *pex5* mutants are compromised in the peroxisomal conversion of IBA  $\beta$ -oxidation to IAA.

JA biosynthesis and perception are required for male fertility (Wasternack and Kombrink, 2010). Although JA biosynthesis is initiated in chloroplasts, the intermediate 12-oxo-phytodienoic acid is transported to the peroxisome for  $\beta$ -oxidation to JA. Measurement of siliques indicated that *pex5-10* makes fewer seeds than the wild type (Fig. 8A). *pex5-1* silique lengths were normal (data not shown). Furthermore, we determined the average seed weight of 100 seeds, and *pex5-10* seeds weighed less than wild-type seeds (Fig. 8B) and frequently looked small and wrinkled (data not shown). However, seed weight of *pex5-10* (*PEX5<sub>454</sub>*) was similar to that in the wild type (Fig. 8B). These data suggest that *pex5-10* has minor fertility defects due to defects in PTS2 import. We also examined the mutants for JA insensitivity by assessing root elongation on medium supplemented with JA. None of the lines exhibited resistance to JA (data not shown).

#### *pex5-10* Has Reduced Peroxisomal Metabolism

Propionate and isobutyrate catabolism happens in peroxisomes using the *CHY1*  $\beta$ -hydroxybutyryl-CoA hydrolase enzyme. Mutants defective in *CHY1* accumulate a toxic intermediate that blocks  $\beta$ -oxidation (Zolman et al., 2001). *chy1* mutants exhibit Suc dependence and IBA/2,4-DB insensitivity (Zolman et al., 2001; Lange et al., 2004; Lucas et al., 2007), similar to other peroxisomal  $\beta$ -oxidation-defective mutants. In addition, the mutant lines have increased sensitivity to root elongation inhibition by propionate and isobutyrate compared with the wild type (Lucas et al., 2007).



**Figure 4.** Fatty acid metabolism in *pex5* mutant seedlings. A, Hypocotyl length of seedlings grown for 24 h in light and 6 d in dark on

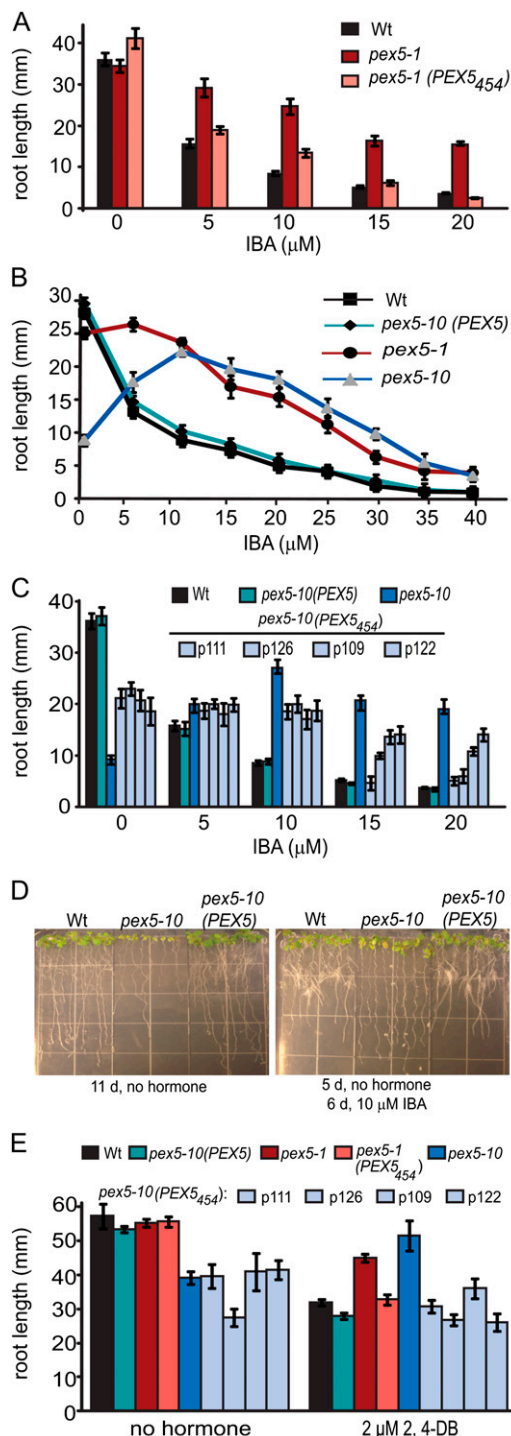
medium supplemented with increasing concentrations of Suc. Error bars represent SE ( $n \geq 12$ ). B, Hypocotyl length of seedlings grown for 24 h in light and 6 d in dark on medium without Suc or with 0.5% Suc. Error bars represent SE ( $n \geq 12$ ). C, Hypocotyl length of seedlings grown for 24 h in light and 6 d in dark on medium without Suc or with 0.5% Suc. *pex5-10* seed coats were nicked immediately after plating. Error bars represent SE ( $n \geq 12$ ). D, Photographs depicting *pex5-10* plant lines. Plants were grown on complete medium for 7 d. *pex5-10* seed coats were nicked after plating. E, Percentage of C20:1 fatty acid (versus total fatty acids) in 6-d-old seedlings grown in light on 0.5% Suc medium. Error bars represent SE of three biological replicates. Wt, Wild type.

To examine if catabolism is compromised in *pex5*, we assayed wild-type and mutant seeds on propionate and isobutyrate. The *chy1* mutant (Zolman et al., 2001) was used as a control and exhibited a high degree of root elongation inhibition from propionate and isobutyrate (Fig. 8C). In contrast, *pex5-1* seedlings showed wild-type responses (Fig. 8C). The *pex5-10* seedlings showed a stronger inhibition on these compounds than the wild type, although the defect was not as severe as in *chy1* (Fig. 8C). Furthermore, the PEX5<sub>454</sub> protein did not rescue this root elongation inhibition (Fig. 8C). These data suggest that these catabolism pathways are affected in the *pex5-10* mutant, and this defect may be based on disruptions in PTS1 protein import.

*pex5-10* seedlings had a pale green color (Fig. 5D). Leaf peroxisomes are involved in photorespiration because they metabolize a nonuseful photosynthetic side product, phosphoglycolate; Arabidopsis mutants with strong defects in photorespiration are a pale green color due to reduced chlorophyll accumulation (Reumann, 2002). We quantified total chlorophyll in both seedlings and adult leaves as an indicator of whether photorespiration was affected in the *pex5* mutants. *pex5-10* showed a reduction in total chlorophyll compared with the wild type (Fig. 8D). This chlorophyll defect was more severe than in the previously described *pex6-1* mutant, which also has defects in PEX5 accumulation (Zolman and Bartel, 2004). The PEX5<sub>454</sub> protein did not rescue the defect in *pex5-10* seedlings (Fig. 8D), and total chlorophyll of *pex5-1* seedlings was similar to that in the wild type (data not shown). These results demonstrate that enzymes in photorespiration require PTS1-mediated import, consistent with the fact that the major photorespiration enzymes each use a PTS1 (Foyer et al., 2009). Interestingly, total chlorophyll in both *pex5-10* and *pex5-10* (PEX5<sub>454</sub>) adult leaves were similar to the wild type, while the *pex6-1* plants maintained this defect (Fig. 8D).

Peroxisomes house many metabolic processes that produce hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which must be detoxified before accumulating to damaging levels. To examine the import of the detoxifying enzymes, we detected H<sub>2</sub>O<sub>2</sub> accumulation in wild-type and mutant leaves using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Thordal-Christensen et al., 1997). Both *pex5-10* and *pex5-10* (PEX5<sub>454</sub>) leaves accumulated a brown precipitate indicative of H<sub>2</sub>O<sub>2</sub> accumulation (Fig. 8E). This

medium supplemented with increasing concentrations of Suc. Error bars represent SE ( $n \geq 12$ ). B, Hypocotyl length of seedlings grown for 24 h in light and 6 d in dark on medium without Suc or with 0.5% Suc. Error bars represent SE ( $n \geq 12$ ). C, Hypocotyl length of seedlings grown for 24 h in light and 6 d in dark on medium without Suc or with 0.5% Suc. *pex5-10* seed coats were nicked immediately after plating. Error bars represent SE ( $n \geq 12$ ). D, Photographs depicting *pex5-10* plant lines. Plants were grown on complete medium for 7 d. *pex5-10* seed coats were nicked after plating. E, Percentage of C20:1 fatty acid (versus total fatty acids) in 6-d-old seedlings grown in light on 0.5% Suc medium. Error bars represent SE of three biological replicates. Wt, Wild type.



**Figure 5.** *pex5* mutant responses to IBA or the IBA analog 2,4-DB. A to C, Root length of 10-d-old seedlings on medium supplemented with IBA. Error bars represent *se* ( $n \geq 12$ ). D, Photographs of 11-d-old wild-type (Wt), *pex5-10*, and *pex5-10 (PEX5)* seedlings grown without hormone or on 10  $\mu$ M IBA. E, Root length of 10-d-old seedlings grown under continuous light on medium supplemented with 2,4-DB. Error bars represent *se* ( $n \geq 12$ ).

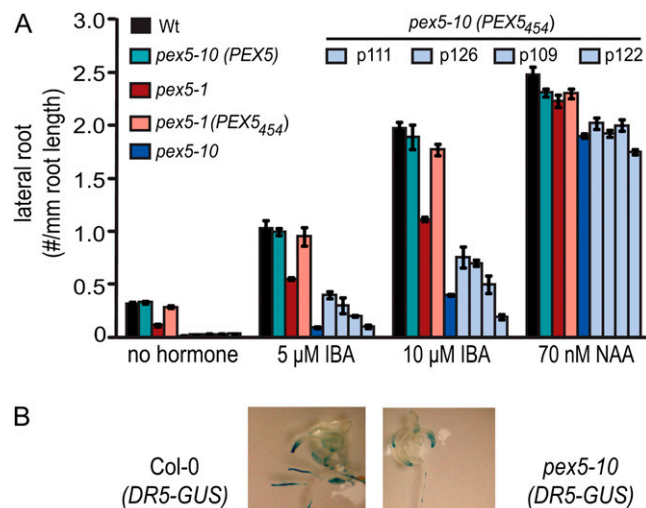
result indicates that  $H_2O_2$  is not efficiently inactivated in *pex5-10*. The PEX5<sub>454</sub> protein only partially rescued the  $H_2O_2$  inactivation defect in *pex5-10* (Fig. 8E), suggesting both PTS1 and PTS2 import pathways are required for detoxification.

***pex5-10* Has Developmental Defects But Normal Morphology**

To determine the consequences of the *pex5-10* insertion, we analyzed plant growth and development. We found initial issues with growth, as evidenced by low root growth and small aerial development (Fig. 5, B and D). *pex5-10* plants showed reduced rosette diameter (Fig. 9, A and B). Once the plants had begun photosynthetic growth, they seemed to recover, and overall plant architecture remained fairly normal. *pex5* and wild-type plants have the same numbers of stems produced, plant height, and date to bolting (Fig. 9C; data not shown).

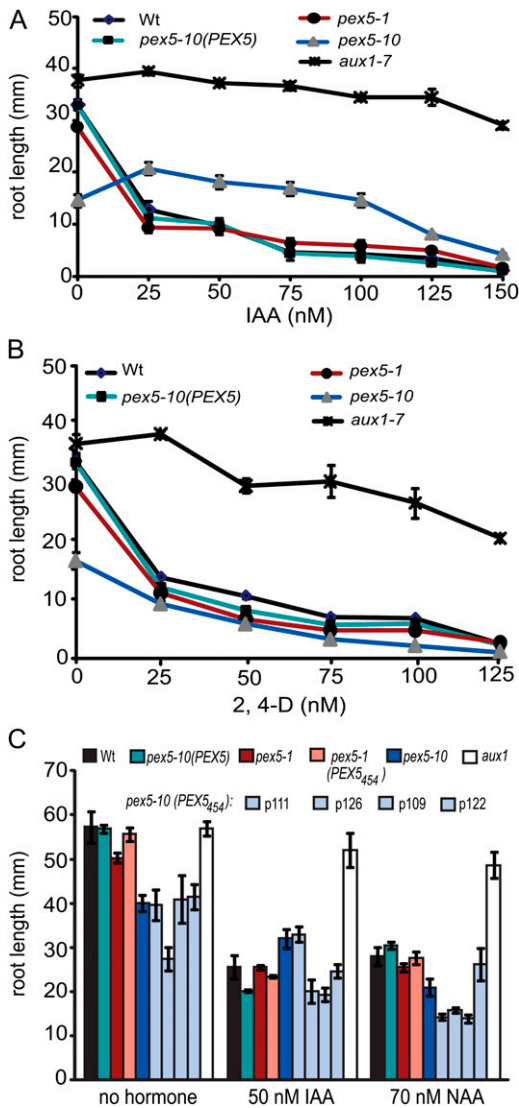
**DISCUSSION**

*pex5-10* is a loss-of-function mutant resulting from a T-DNA insertion in the *PEX5* gene. The isolation of this mutant was unexpected; our original hypothesis was that a *pex5* insertion mutant would be embryo lethal, similar to several other peroxisome-defective mutants (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005). When we analyzed the effect of the insertion at the genetic and protein levels,



**Figure 6.** *pex5* lateral root formation. A, Lateral root density of seedlings grown without hormone for 5 d and then transferred to medium with or without hormone for another 5 d. Data are shown as the number of lateral roots per mm root length. Error bars represent *se* ( $n \geq 12$ ). B, *DR5-GUS* expression in 8-d-old Col-0 and *pex5-10* transgenic seedlings grown on complete medium with 0.5% Suc. Wt, Wild type.

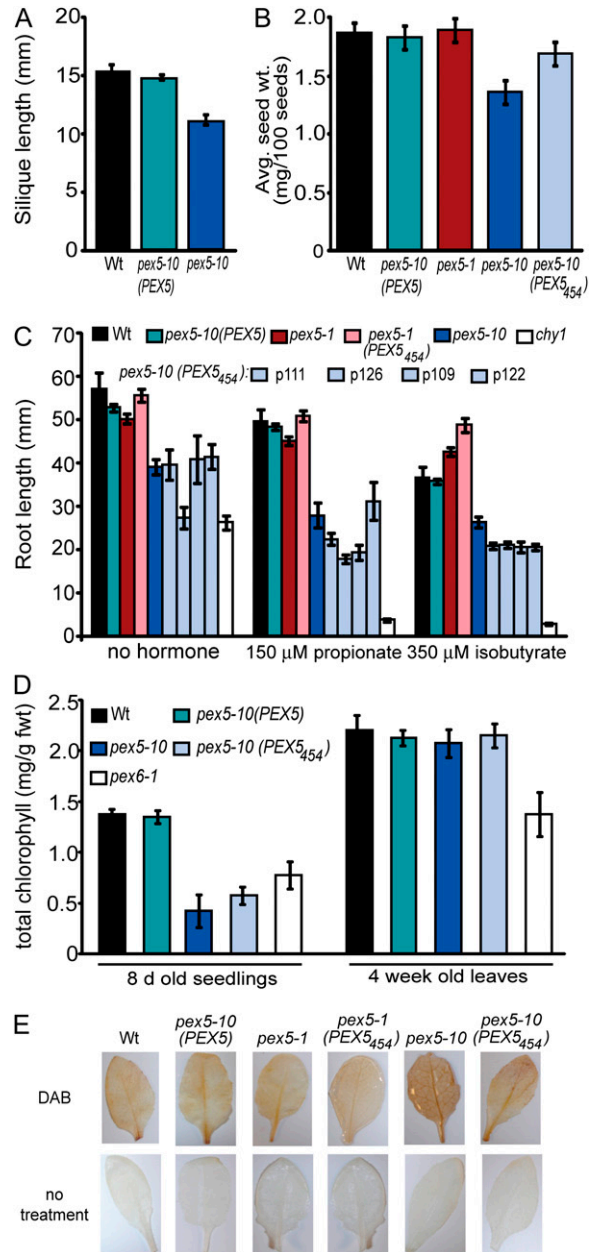




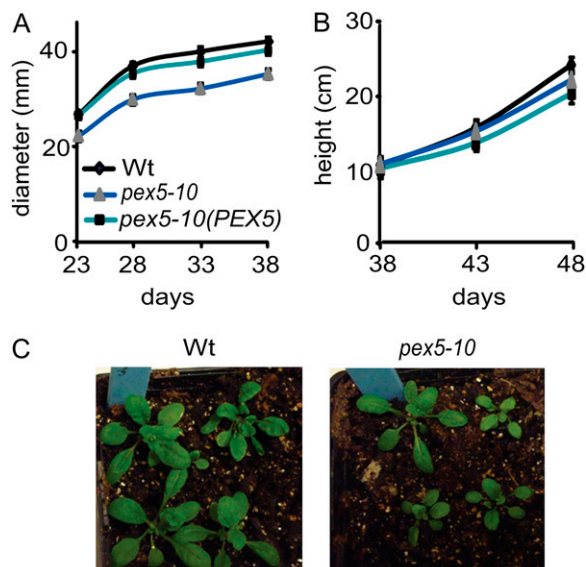
**Figure 7.** *pex5* mutant responses to IAA, 2,4-D, and NAA. Root length is shown for 10-d-old seedlings grown on medium with IAA (A and C), 2,4-D (B), or NAA (C). The auxin transport mutant *aux1* (Pickett et al., 1990) was used as a control to demonstrate insensitivity to IAA. Error bars represent SE ( $n \geq 12$ ). Wt, Wild type.

however, we found that the insertion effects were atypical. The insertion does lead to a loss of gene expression, as seen using primers in the exon in which the insertion is located (Fig. 2C). However, at some frequency, this exon and the insertion are removed in the Salk T-DNA line (Fig. 2D). This excision results in a protein with a deletion (Fig. 2, A and B); the *pex5-10* mutant protein is missing four of the first five PPRs (Fig. 1A). In addition to the deletion, the mutant protein is at a reduced concentration compared with the wild-type protein (Fig. 2A). Although this excision does occur at the normal splicing boundaries, we found no evidence for this splice form in the wild type, either in the cDNA collection or in our RT-PCR trials (data not shown). In monocots, a PEX5 short form (PEX5pS) has been iden-

tified, which also is missing an exon compared with the full-length long form (PEX5pL); however, the *pex5-10* removal of exon 5 does not correspond to the exon 7 deletion in rice (*Oryza sativa*), and the *pex5-10* and PEX5pS alterations affect different domains in the protein (Lee et al., 2006).



**Figure 8.** *pex5-10* fertility, photorespiration, and H<sub>2</sub>O<sub>2</sub> inactivation defects. A, Length of the fifth silique on the main stem. Error bars represent SE ( $n \geq 18$ ). B, Average seed weight in mg per 100 seeds. Error bars represent SE ( $n \geq 4$ ). C, Root length of 10-d-old seedlings grown on medium containing propionate or isobutyrate. Error bars represent SE ( $n \geq 12$ ). D, Total chlorophyll content in mg per g fresh weight for 8-d-old seedlings and 4-week-old leaves. Error bars represent SE ( $n \geq 6$ ). E, DAB staining showing the accumulation of H<sub>2</sub>O<sub>2</sub> in 2-week-old leaves. Wt, Wild type.



**Figure 9.** *pex5-10* growth and development. A and B, Wild-type (Wt), *pex5-10* (*PEX5*), and *pex5-10* plants were grown under continuous white light at 22°C with normal watering two times per week. Plants were measured for rosette diameter (A) and plant height (B) on the indicated days. Error bars represent se ( $n \geq 18$ ). C, Photographs of 3-week-old soil-grown wild-type and *pex5-10* plants.

This type of response by the plant to a T-DNA insertion is rare; a global analysis of the effects of insertions in exons revealed that only 2% of those studied resulted in truncated proteins being formed, compared with 88% of the samples with a loss of protein accumulation (Wang, 2008). However, isolation of this splicing event in the insertion mutant was fortuitous, as it allowed the study of PEX5 function when a true *pex5* null likely would be embryo lethal. The mutant *pex5-10* protein must retain just enough activity to maintain the mutant plant growth and development, particularly in adult plants. The defects in *pex5-10* are stronger than *pex5-1* but fall in a similar spectrum. This allelic spectrum allows us to draw conclusions about the function of PEX5 in plant growth and development. PEX5 is clearly required for the import of peroxisome matrix proteins, as *pex5-10* showed mislocalization of both PTS1- and PTS2-containing GFP reporters (Fig. 3A), consistent with previous studies (Lee et al., 2006; Lingard et al., 2009). In addition, *pex5-10* has altered localization of thiolase (Fig. 3C; Zolman et al., 2005; Lingard and Bartel, 2009; Ramón and Bartel, 2010), catalase (Lingard and Bartel, 2009; Lingard et al., 2009), isocitrate lyase (Lingard et al., 2009), malate synthase (Lingard and Bartel, 2009), and peroxisomal malate dehydrogenase (Lingard and Bartel, 2009; Ramón and Bartel, 2010). However, the *pex5-10* mutant is able to correctly localize the peroxisomal membrane protein PEX14 (Lingard and Bartel, 2009), indicating that peroxisomal structures are intact and that membrane protein import occurs normally. This loss of matrix import can be seen in

reduced peroxisomal matrix protein enzymatic activity (Fig. 3D). Therefore, this T-DNA mutant has enabled us to score the effects of loss of peroxisomal function, as the mutant strongly disrupts all peroxisome matrix processes by decreased import of the required proteins.

Despite the numerous functions of peroxisomes throughout the plant life cycle (Kaur et al., 2009), perhaps PEX5 is most critical in early seedlings, when peroxisomal fatty acid  $\beta$ -oxidation is required for development. The *pex5-10* mutant has a low degree of germination (0%–20%) without assistance (Tables I and II) and a strict requirement for exogenous Suc during seedling development (Fig. 4; Lee et al., 2006, 2007; Lingard and Bartel, 2009; Ramón and Bartel, 2010). The requirement for seed coat disruption by nicking is similar to several other strong peroxisome-defective mutants (Footitt et al., 2002; Pracharoenwattana et al., 2005; Hooks et al., 2007; Kanai et al., 2010). Despite severe germination and seedling developmental phenotypes, some mutant seedlings survive and develop into adult plants. Seedlings also are unable to metabolize IBA/2,4-DB, resulting in alterations in secondary root development (Figs. 5 and 6; Lee et al., 2006, 2007; Lingard and Bartel, 2009; Ramón and Bartel, 2010). As plants develop and start photosynthesis, PEX5 decreases (Fig. 1B). This trend may explain the strong seedling defects in *pex5-10* seedlings but less severe phenotypes of the adult plants (Fig. 9). Our enzymatic studies of peroxisomal processes (Fig. 3D) and examination of  $\beta$ -oxidation using eicosenoic acid as a marker (Fig. 4E) demonstrate the effects of decreased peroxisomal import in *pex5-10* and explain the above defects in plant development. Hayashi et al. (2005) used RNA interference to decrease *PEX5* levels, resulting in a dwarf phenotype and photorespiration defects. In addition, the silenced lines were Suc dependent and resistant to 2,4-DB, with defects in both PTS1 and PTS2 import (Hayashi et al., 2005). These phenotypes are similar to *pex5-10*. Each of these defective processes reveals the importance of PEX5 in peroxisomal function and the various roles of peroxisomes in plant growth and development.

Although *pex5-1* and *pex5-10* mutants allowed us to study defects in peroxisomal matrix protein import, neither was appropriate to specifically study PTS1 import. We wanted to generate a line defective specifically in PTS1 import so we could study the consequences of disruptions in PTS1 and PTS2 import separately. The *pex5-10* (*PEX5*<sub>454</sub>) construct expresses the N-terminal region of PEX5, including the PPR domains required for binding to peroxisomal membrane proteins and the PEX7-binding domain. These domains should allow normal peroxisomal import for the PEX7-bound PTS2 proteins via PEX5. However, PEX5<sub>454</sub> is missing the TPR domains required for binding the tripeptide PTS1 at the C terminus of the cargo protein. We crossed this construct into the *pex5-1* and *pex5-10* mutants. We hypothesized that PEX5<sub>454</sub> would restore *pex5-1* defects and partially complement

*pex5-10*. Indeed, PTS2-GFP import was restored in both *pex5* transgenic lines to a wild-type pattern (Fig. 3A; data not shown). However, GFP-PTS1 remained cytosolic in *pex5-10* (*PEX5<sub>454</sub>*), similar to the mutant parent lines (Fig. 3A). Although *pex5-1* and *pex5-10* retain much of thiolase (a PTS2 model protein) in the unprocessed, cytosolic form, the transgenic lines exhibited increased peroxisomal import (Fig. 3C). In addition, *PEX5<sub>454</sub>* restored the activity of the peroxisomally localized ACX enzymes. ACX4 has a PTS1 and is known to be the primary enzyme required for the oxidation of short-chain fatty acid substrates (Hayashi et al., 1999). The *pex5* mutants cannot oxidize C6:0 substrates, a defect that is not affected by insertion of the *PEX5<sub>454</sub>* construct (Fig. 3D). ACX3 is the major enzyme affecting C12:0 oxidation; this enzyme has a PTS2 (Eastmond et al., 2000; Froman et al., 2000). ACX3 activity is reduced in both mutants but is recovered partially in the transgenic lines (Fig. 3D), indicating that the *PEX5<sub>454</sub>* protein is increasing PTS2 responses. Therefore, the defects in *pex5-10* (*PEX5<sub>454</sub>*) result from a specific defect in PTS1 import.

Our examination of the *pex5* and *pex5* (*PEX5<sub>454</sub>*) lines allowed us to separate which import pathways are required for several peroxisomal processes (summarized in Supplemental Table S1). Comparison of the *pex5-1* mutant and the *pex5-1* (*PEX5<sub>454</sub>*) lines reveals a fairly complete phenotypic rescue under all conditions, consistent with the idea that the *pex5-1* defect primarily disrupts PTS2 import and that *PEX5<sub>454</sub>* restores PTS2 activity. In the *pex5-1* background, the *PEX5<sub>454</sub>*-mediated recovery of PTS2 activity is evident with increased seed storage fatty acid utilization (Fig. 4E), increased development in the absence of Suc (Fig. 4B), and responses to IBA (Figs. 5A and 6A). Similarly, the *pex5-10* (*PEX5<sub>454</sub>*) plants show improved growth and development, although the transgenic lines remain impaired. Expression of *PEX5<sub>454</sub>* in *pex5-10* promotes increased germination without physical stimuli compared with *pex5-10*, although substantial delays remain compared with the wild type (Table I). Improvements also include an increased ability to grow without Suc supplementation (Fig. 4, B–D), higher  $\beta$ -oxidation rates (Fig. 4E), greater responses to IBA stimuli (Figs. 5C and 6A), and an improved ability to make seeds (Fig. 8B). *pex5-10* (*PEX5<sub>454</sub>*) plants show only a partial rescue of the *pex5-10* phenotypes, consistent with the idea that PTS1 import still is disrupted in these lines. Although the import of PTS2-containing proteins restores some peroxisomal processes, most pathways require enzymes with both PTS1 and PTS2 signals.

Interestingly, two assays did not show any changes between the mutant parent and the *PEX5<sub>454</sub>* transgenic line, allowing us to speculate about the types of import required. First, an inability of plants to metabolize either propionate and isobutyrate results in a hypersensitive phenotype, likely due to the accumulation of toxic intermediates in the pathway that inhibit growth (Lucas et al., 2007). *pex5-10* was sensitive to both of these compounds, which was not affected by *PEX5<sub>454</sub>*

expression (Fig. 8C). This result suggests that the enzymes (or at least the key rate-limiting enzymes) involved in processing these metabolites have a PTS1. This mechanism is consistent with the suggested enzymatic pathways (Lucas et al., 2007). Also, photorespiration rates were essentially the same in *pex5-10* and *pex5-10* (*PEX5<sub>454</sub>*) lines (Fig. 8D). The four key peroxisomal enzymes involved in photorespiration each use PTS1 import pathways. The fact that these defects are not affected by *PEX5<sub>454</sub>* expression and, therefore, may specifically require PTS1 import is further supported by the result that *pex5-1* does not exhibit a defective phenotype in any of these assays. In contrast, responses to 2,4-DB (Fig. 5E) were completely rescued by *PEX5<sub>454</sub>* expression, revealing that the disruption in *pex5-10* may be caused by mislocalization of a key PTS2-containing protein. A recent study indicated that 2,4-DB  $\beta$ -oxidation to 2,4-D may occur using a distinct and/or overlapping set of enzymes as IBA  $\beta$ -oxidation to IAA, although AAE18 is the only protein with known differential activity and this enzyme has a PTS1 (Wiszniewski et al., 2009).

In this paper, we determined the effect of the *pex5-10* mutation on *PEX5* gene expression and protein accumulation. We then examined the importance and function of *PEX5* in peroxisomal targeting by observing the phenotypes of *pex5* mutants. The defects shown here confirm the critical role of *PEX5* in peroxisomal metabolism. Additional comparisons of the three *pex5* lines will be useful for enhanced studies of the protein complements of plant peroxisomes and for studies of the mechanism of plant peroxisomal matrix protein import.

## MATERIALS AND METHODS

### Plant Material

*Arabidopsis* (*Arabidopsis thaliana*) *pex5-1* has been previously published with a Ser-to-Leu mutation at amino acid 318 in At5g56290 (Zolman et al., 2000). *pex5-10* (SALK\_124577) was obtained from the Salk collection of insertion mutants (Alonso et al., 2003) and was backcrossed at least twice prior to analyses. The mutant can be tracked experimentally using PCR amplification with a combination of genome-specific and insertion-specific primer combinations (Supplemental Table S2). *chy1-3* (At5g65940; Zolman et al., 2001), *pex6-1* (At1g03000; Zolman and Bartel, 2004), and *aux1-7* (At2g38120; Pickett et al., 1990) were used as controls. All mutants are in the Col-0 background.

### Growth Conditions and Phenotypic Assays

Seeds were surface sterilized with 30% bleach:0.1% Triton solution for 10 min and rinsed four times with sterile water. All seedling assays were done on plates wrapped with gas-permeable Micropore surgical tape, incubated at 22°C under continuous light for the indicated number of days. If plates contained auxins, yellow-filtered light was used (Stasinopoulos and Hangarter, 1990). Plants grown to maturity were placed in pots of Sun-Gro Metro Mix 360 soil and incubated at 22°C to 25°C under continuous white light.

For germination assays, 100 seeds were plated on solid plant nutrient medium (PN; Haughn and Somerville, 1986) supplemented with 0.5% Suc (PNS). F2 seeds were the second (segregating) generation of a backcross to the Col-0 parent. Seeds were incubated at 22°C under continuous white light. Plates were examined with a dissecting microscope for the number of seeds that germinated every 24 h for 4 d. Seeds that did not germinate by day 4 were transferred in a sterile manner to a fresh PNS plate, and the seed coats were

nicked using a Leica Zoom 2000 dissecting microscope with sterile forceps. After 10 d, DNA was extracted from all seedlings. The genotype of each seedling was determined by PCR amplification with gene-specific PEX5 primers and T-DNA primers (Supplemental Table S1).

To assay the Suc dependence of *pex5-10*, seeds were plated on PN medium alone or on medium supplemented with different concentrations of Suc. After plating, seeds were nicked with sterile forceps and then incubated at 22°C under continuous white light for 24 h. Plates were transferred to the dark for 6 d and hypocotyl length was measured.

For root elongation assays, seeds were plated on PNS alone or with the indicated hormone concentrations. After plating, all *pex5-10* seeds were nicked. After 10 d, seedlings were removed from plates and root length was measured.

To determine lateral root initiation, seeds were plated on PNS medium and *pex5-10* seeds were nicked. After 5 d, seedlings were transferred to fresh PNS plates supplemented with or without hormone and incubated for an additional 5 d. The number of lateral roots was counted with a Leica Zoom 2000 dissecting microscope, and the root length measured. Data are represented as lateral root density or lateral root number per root length (mm).

For the adult phenotypic assays, seeds were surface sterilized and plated on solid PNS medium. *pex5-10* seeds were nicked immediately after plating. Ten-day-old seedlings were transferred to soil. Plants were watered once weekly, and measurements were taken at regular intervals for rosette diameter, number of stems, and plant height. Each plant was observed daily for date of bolting. The fifth silique from the main stem was measured for each plant. The weight of 100 wild-type and mutant seeds was recorded, and photographs were taken.

## RNA Isolation and RT-PCR Analysis

To examine the effects of the T-DNA insertion on *PEX5* gene expression, we isolated RNA from wild-type and 3-week-old mutant plants. Total RNA was isolated with the RNeasy Plant Kit (Qiagen). RNA was treated with DNase (Fisher Scientific) to remove contaminating genomic DNA, and cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen). One microliter of cDNA was used for PCR amplification with gene-specific primer sets (Supplemental Table S2). For all PCR amplifications, Col-0 genomic DNA was used as a control. PCR products were resolved on a 2% agarose gel.

## GFP Examination of Peroxisomal Import

To examine the import of proteins into peroxisomes, we used the GFP-PTS1 construct, which includes the SKL import signal at the C terminus of the GFP (Zolman and Bartel, 2004), and PTS2-GFP, which contains the targeting signal from the Arabidopsis PED1/KAT2 thiolase enzyme fused to the N terminus of GFP (Woodward and Bartel, 2005). The GFP parent lines in the Col-0 background were crossed to the mutant lines, and plants homozygous for the appropriate mutation and construct combinations were identified by PCR and immunoblotting. GFP analysis was done on root hair cells using a Zeiss LSM700 confocal microscope.

## Protein Extraction and Immunoblot Analysis

For western-blot analysis, samples were ground on dry ice and mixed with 2× NuPAGE LDS Sample Buffer (Invitrogen). The mixture was centrifuged, and the supernatant containing the protein was collected. Protein was quantified with Bradford reagent (Sigma-Aldrich), and 40 μg of total protein was electrophoresed on NuPAGE 10% Bis-Tris gels with NuPAGE MOPS SDS Running Buffer (Invitrogen). Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 2 h in 10% (w/v) nonfat dry milk in Tris-buffered saline plus Tween 20 buffer. Membranes were incubated with primary antibodies recognizing the C-terminal region of PEX5 (Zolman and Bartel, 2004; 1:1,000 dilution), the myc epitope (Sigma; 1:1,000), thiolase (Lingard et al., 2009; 1:5,000), or HSC70 (Stressgen Bioreagents; 1:5,000). Secondary detection involved horseradish peroxidase-linked anti-rabbit antibodies (PEX5 and PED1; Southern Biotech; 1:10,000) and rabbit anti-mouse antibodies (myc and HSC70; Sigma; 1:10,000). All antibodies were visualized using SuperSignal West Dura extended duration substrate (Thermo Scientific).

## Fatty Acid Extraction and GC Analysis of C20:1 Content

Seeds were surface sterilized, plated on solid PNS medium covered with filter paper, and incubated for 6 d. Fatty acid extraction and esterification were

done (Devaiah et al., 2007) using 50 seedlings. Prior to esterification, 50 μM heptadecanoic acid (C17:0) was added to each sample as an internal standard. Analysis was done using an automated Shimadzu gas chromatograph (Shimadzu GC-17A) equipped with a silica capillary column (DB-5MS, 30 m, 0.25 mm, 0.25 μm). The temperatures of the injector column and flame ionization detector were 220°C, 170°C, and 220°C.

## DAB and DR5-GUS Staining

To visualize the accumulation of H<sub>2</sub>O<sub>2</sub>, we used DAB stain, which reacts with H<sub>2</sub>O<sub>2</sub> to produce a brown precipitate (Thordal-Christensen et al., 1997). Briefly, leaves were collected from 2-week-old plants and vacuum infiltrated with 1 mg mL<sup>-1</sup> DAB solubilized in Tris-acetate buffer, pH 3. Following infiltration, leaves were incubated with the stain for 24 h in the dark, then boiled in 80% ethanol to remove chlorophyll for visualization.

For DR5-GUS visualization, Col-0 and *pex5-10* transgenic seedlings were stained at 37°C for 2 d in 0.5 mg mL<sup>-1</sup> 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Bartel and Fink, 1994).

## Chlorophyll Quantification

Chlorophyll extraction and quantification were performed using modifications of a previously published protocol (Wintermans and de Mots, 1965). Five milligrams of 8-d-old seedlings or 4-week-old leaves was boiled in 96% ethanol at 80°C until all the chlorophyll was removed. The chlorophyll content was measured with a spectrophotometer at 654, 665, and 649 nm, and total chlorophyll content was calculated.

## PEX5<sub>454</sub> Transgenic Plants

The 35S-*PEX5* overexpression construct has been described previously (Zolman et al., 2000). *pex5* mutant lines transformed with this construct can be selected by resistance to the herbicide Basta.

We created a truncated gene construct of the first 454 amino acids of the PEX5 protein. The truncated *PEX5* cDNA was amplified with primers PEX5-*Sal* and PEX5 454 B/N (Supplemental Table S2) using Ex-Taq (TaKaRa), the PCR product was run on a 1% guanosine gel, and the DNA was purified with the Qiaex II Gel Extraction Kit (Qiagen). The purified product was ligated into a TOPO vector (Invitrogen), and the insert was verified by sequencing prior to subcloning into pBluescript KS+ vector (pKS) using *Sall* and *NotI*. For myc ligation to PEX5<sub>454</sub>, pT26 myc and pKS PEX5<sub>454</sub> were digested with *Bam*HI. The pT26 myc digestion product was electrophoresed, and the myc fragment was extracted. The *Bam*HI-digested pKS PEX5<sub>454</sub> was dephosphorylated using SAP (Promega). The myc fragment was ligated at the C terminus of the PEX5<sub>454</sub> gene. For cloning of the PEX5<sub>454</sub> insert (with the myc epitope) into the plant transformation vector 35S pBARN (LeClere and Bartel, 2001), pKS PEX5<sub>454</sub> was digested with *Apa*I and blunt ends were created using T4 DNA polymerase (New England Biolabs) followed by digestion with *NotI*. 35S pBARN was digested with *Sma*I and *NotI*. The 35S-PEX5<sub>454</sub> construct was transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) and transformed into wild-type plants (Clough and Bent, 1998). Transformants were selected by Basta resistance. The Col-0 (PEX5<sub>454</sub>) line was crossed to *pex5-1* and *pex5-10*, which were selected first by genotyping the mutation and then by identifying lines containing the cDNA.

## ACX Enzyme Assays

Col-0 and mutant seeds were surface sterilized and plated on solid PNS medium covered with filter paper. ACX assays were performed similar to previously described protocols (Hryb and Hogg, 1979; Gerhardt, 1987; Adham et al., 2005). A total of 0.3 g of 4-d-old seedlings was immersed in liquid nitrogen and ground to powder in a mortar. Ground seedlings were incubated for 15 min at 4°C in 800 μL of cold extraction buffer containing 50 mM KPO<sub>4</sub>, 50 μM FAD, and 0.01% Triton X-100 supplemented with 10 μL of protease inhibitor cocktail (Sigma). After incubation, extracts were centrifuged for 10 min at 13,200 rpm, and the supernatant was loaded on quick-spin protein columns (Roche) and processed according to the manufacturer's instructions. For the enzymatic reaction, 200 μL of extract was added to 200 μL of reaction mix (50 mM KPO<sub>4</sub>, 0.025% Triton X-100, 50 mM *p*-hydroxybenzoic acid, 100 μg mg<sup>-1</sup> bovine serum albumin, 50 μM FAD, 2 mM 4-aminoantipyrine, and 110 units of horseradish peroxidase) and 125 μM C6:0 and C12:0 substrates (Sigma). The reaction was measured spectrophotometrically at 500 nm for

H<sub>2</sub>O<sub>2</sub> production, and a standard H<sub>2</sub>O<sub>2</sub> curve was created to calculate H<sub>2</sub>O<sub>2</sub> concentration.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Table S1.** Summary of phenotypes in *pex5* mutant lines.

**Supplemental Table S2.** Primers used in this work.

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