Running head: LON2 facilitates peroxisome matrix protein import

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Arabidopsis LON2 is necessary for peroxisomal function and sustained matrix protein import

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ABSTRACT

Relatively little is known about the small subset of peroxisomal proteins with predicted protease activity. Here we report that the peroxisomal LON2 (At5g47040) protease facilitates matrix protein import into Arabidopsis thaliana peroxisomes. We identified T-DNA insertion alleles disrupted in five of the nine confirmed or predicted peroxisomal proteases and found only two – lon2 and deg15, a mutant defective in the previously described PTS2-processing protease (DEG15/At1g28320) – with phenotypes suggestive of peroxisome metabolism defects. Both lon2 and deg15 mutants were mildly resistant to the inhibitory effects of indole-3-butyric acid (IBA) on root elongation, but only lon2 mutants were resistant to the stimulatory effects of IBA on lateral root production or displayed sucrose-dependence during seedling growth. lon2 mutants displayed defects in removing the type 2 peroxisome targeting signal (PTS2) from peroxisomal malate dehydrogenase and reduced accumulation of 3-ketoacyl-CoA thiolase, another PTS2-containing protein; both defects were not apparent upon germination but appeared in 5- to 8-day-old seedlings. In lon2 cotyledon cells, matrix proteins were localized to peroxisomes in 4-day-old seedlings, but mislocalized to the cytosol in 8-day-old seedlings. Moreover, a PTS2-GFP reporter sorted to peroxisomes in lon2 root tip cells, but was largely cytosolic in more mature root cells. Our results indicate that LON2 is needed for sustained matrix protein import into peroxisomes. The delayed onset of matrix protein sorting defects may account for the relatively weak sucrose dependence following germination, moderate IBA-resistant primary root elongation, and severe defects in IBA-induced lateral root formation observed in lon2 mutants.
INTRODUCTION

Peroxisomes are single-membrane-bound organelles found in most eukaryotes. Peroxin (PEX) proteins are necessary for various aspects of peroxisome biogenesis, including matrix protein import (reviewed in Distel et al., 1996; Schrader and Fahimi, 2008). Most matrix proteins are imported into peroxisomes from the cytosol using one of two targeting signals, a C-terminal type 1 peroxisome-targeting signal (PTS1) or a cleavable N-terminal type 2 peroxisome-targeting signal (PTS2) (Reumann, 2004). PTS1- and PTS2-containing proteins are bound in the cytosol by soluble matrix protein receptors, escorted to the peroxisome membrane docking complex, and translocated into the peroxisome matrix (reviewed in Platta and Erdmann, 2007). Once in the peroxisome, many matrix proteins participate in metabolic pathways, such as \( \beta \)-oxidation, hydrogen peroxide decomposition, and photorespiration (reviewed in Gabaldon et al., 2006; Poirier et al., 2006).

In addition to metabolic enzymes, several proteases are found in the peroxisome matrix. Only one protease, DEG15/Tysnd1, has a well-defined role in peroxisome biology. The rat Tysnd1 protease removes the targeting signal after PTS2-containing proteins enter the peroxisome and also processes certain PTS1-containing \( \beta \)-oxidation enzymes (Kurochkin et al., 2007). Similarly, the Arabidopsis Tysnd1 homolog DEG15 (At1g28320) is a peroxisomal serine protease that removes PTS2 targeting signals (Helm et al., 2007; Schuhmann et al., 2008).

In contrast to DEG15, little is known about the other eight Arabidopsis proteins that are annotated as proteases in the AraPerox database of putative peroxisomal proteins (Reumann et al., 2004) (Table I). Two of these proteins, At4g14570 and At4g36195, have been identified in vacuolar proteomics studies (Carter et al., 2004; Shimaoka et al., 2004), which, in combination with the “minor” PTS found in both of these predicted proteases (Reumann, 2004), suggests that these enzymes may not be peroxisomal. Along with DEG15, only two of the predicted peroxisomal proteases, an M16 metalloprotease (At2g41790), which we have named PXM16 for peroxisomal M16 protease, and a Lon-related protease (At5g47040/LON2, Ostersetzer et al., 2007), are found in the proteome of peroxisomes purified from Arabidopsis suspension cells (Eubel et al., 2008). DEG15 and LON2 also have been validated as peroxisomally targeted using GFP fusions (Ostersetzer et al., 2007; Schuhmann et al., 2008).

PXM16 is the only one of the nine Arabidopsis M16 (pitrilysin family) metalloproteases (García-Lorenzo et al., 2006; Rawlings et al., 2008) containing a predicted PTS. M16 subfamilies B and C contain the plastid and mitochondrial processing peptidases (reviewed in Schaller, 2004), whereas PXM16 belongs to M16 subfamily A, which includes insulin-degrading peptidases (Schaller, 2004). A tomato M16 subfamily A protease similar to insulin-degrading enzymes with a putative PTS1 was identified in a screen for proteases that cleave the wound-
response peptide hormone systemin (Strassner et al., 2002), but the role of Arabidopsis PXM16 is unknown.

Arabidopsis LON2 is a typical Lon protease with three conserved domains; an N-terminal domain, a central ATPase domain in the AAA family, and a C-terminal protease domain with a serine-lysine catalytic dyad (Fig. 1A and Lee and Suzuki, 2008). Lon proteases are found in prokaryotes and in some eukaryotic organelles (Fig. 1C) and participate in protein quality control by cleaving unfolded proteins and can regulate metabolism by controlling levels of enzymes from many pathways, including cell cycle, metabolism, and stress responses (reviewed in Tsilibaris et al., 2006). Four Lon homologs are encoded in the Arabidopsis genome; isoforms have been identified in mitochondria, plastids, and peroxisomes (Ostersetzer et al., 2007; Eubel et al., 2008; Rawlings et al., 2008). Mitochondrial Lon proteases are found in a variety of eukaryotes and function both as ATP-dependent proteases and as chaperones promoting protein complex assemblies (Fig. 1A and Lee and Suzuki, 2008). LON2 is the only Arabidopsis Lon isoform with a canonical C-terminal PTS1 (SKL-COOH) (Ostersetzer et al., 2007) or found in the peroxisome proteome (Eubel et al., 2008; Reumann et al., 2009). Functional studies have been conducted with peroxisomal Lon isoforms found in the proteome of peroxisomes purified from rat hepatic cells (pLon, Kikuchi et al., 2004) and the methylotrophic yeast Hansenula polymorpha (Pln, Aksam et al., 2007). Rat pLon interacts with β-oxidation enzymes, and a cell line expressing a dominant negative pLon variant has decreased β-oxidation activity, displays defects in the activation processing of PTS1-containing acyl-CoA oxidase, and missorts catalase to the cytosol (Omi et al., 2008). H. polymorpha Pln is necessary for degradation of a misfolded, peroxisome-targeted version of dihydrofolate reductase and for degradation of in vitro-synthesized alcohol oxidase in peroxisomal matrix extracts, but does not contribute to degradation of peroxisomally-targeted GFP (Aksam et al., 2007).

In this work, we examined the roles of several putative peroxisomal proteases in Arabidopsis. We found that lon2 mutants displayed peroxisome-deficient phenotypes, including resistance to the protoauxin indole-3-butyric acid (IBA) and age-dependent defects in peroxisomal import of PTS1- and PTS2-targeted matrix proteins. Our results indicate that LON2 contributes to matrix protein import into Arabidopsis peroxisomes.

RESULTS

LON2 Is Necessary for IBA-Stimulated Lateral Root Formation

We analyzed mutants defective in predicted peroxisomal proteases to assess contributions of the proteases to peroxisome functions. pxm16-1 and pxm16-2 have T-DNA insertions in exons 1 and 2 of PXM16, respectively; lon2-1 and lon2-2 carry independent insertions in the final LON2 exon (Fig. 1B). The lon2-1 and lon2-2 T-DNAs may allow LON2 expression; however, the
tron2-1 and lon2-2 products would lack the C-terminal PTS1 and likely mislocalize to the cytosol. The previously described T-DNA insertion in the fifth intron of DEG15 (deg15-1) (Helm et al., 2007; Schuhmann et al., 2008) was included for comparison (Fig. 1B).

To determine whether the protease mutations impaired peroxisomal fatty acid β-oxidation, we assessed seedling hypocotyl elongation in the dark and root elongation in the light with and without supplemental sucrose. Peroxisome biogenesis mutants often display defects in peroxisomal β-oxidation of fatty acids stored in seeds and consequently require sucrose during early seedling development (Hayashi et al., 1998; Zolman et al., 2000). We compared the protease mutants to pex5-10 and pex6-1, sucrose dependent peroxin mutants (Zolman and Bartel, 2004; Zolman et al., 2005), and ped1-96, a likely null allele that we identified (M.I.L and B.B., unpublished) in the gene encoding the predominant seedling 3-ketoacyl-CoA thiolase isozyme (Germain et al., 2001). Like other ped1/kat2 alleles in different accessions (Hayashi et al., 1998; Germain et al., 2001), the ped1-96 mutant exhibited severe hypocotyl elongation defects in the dark (Fig. 2, A and B) and root elongation defects in the light (Fig. 2, C and D) in the absence of sucrose supplementation.

As previously reported (Schuhmann et al., 2008), we found that the deg15-1 mutant, like wild type, had long hypocotyls and roots regardless of presence or absence of sucrose (Fig. 2). Similarly, pxm16-1 and pxm16-2 displayed sucrose-independent hypocotyl and root elongation (Fig. 2). By contrast, although the lon2 mutant hypocotyls were not significantly sucrose dependent in the dark, lon2-1 and lon2-2 displayed root elongation defects in the light that were partially restored by sucrose (Fig. 2).

Many pex mutants and other β-oxidation mutants display apparent defects in converting the protoauxin indole-3-butyric acid (IBA) to the active auxin indole-3-acetic acid (IAA) and therefore are resistant to both the inhibitory effects of IBA on root elongation and to the stimulatory effects of IBA on lateral root production (Zolman et al., 2000; Zolman and Bartel, 2004; Adham et al., 2005; Woodward and Bartel, 2005; Zolman et al., 2005). We found that pxm16-1 and pxm16-2 resembled wild type in the root elongation assay, and that lon2-1, lon2-2, and deg15-1 were mildly resistant to low IBA concentrations compared to the strong IBA resistance of ped1-96, pex5-10, and pex6-1 (Fig. 3, A and B).

In contrast to the mild IBA resistance exhibited by lon2 mutants when assayed for root elongation inhibition (Fig. 3, A and B), lon2-1 and lon2-2 seedlings were both dramatically resistant to the stimulatory effects of IBA on lateral root formation, similar to ped1-96 and pex6-1 seedlings (Fig. 3C). In contrast, deg15 and pxm16 mutants resembled wild type in the lateral root assay, efficiently producing lateral roots in response to IBA (Fig. 3C). All lines produced lateral roots when grown on the synthetic auxin 1-naphthaleneacetic acid (NAA), suggesting that the lateral-root deficiency in the lon2 mutants stemmed from defective
peroxisomal IBA metabolism, rather than a general inability to produce lateral roots or respond to auxin. The striking lon2 resistance to IBA-stimulated lateral root formation, accompanied by only mild resistance to the inhibitory effects of IBA on primary root elongation, suggested that LON2 may be more important for peroxisomal IBA metabolism as root cells mature and differentiate.

In addition to the defects displayed in specific assays of peroxisomal function described above, lon2-1 and lon2-2 mutants were smaller than wild type both as seedlings (Fig. 2D) and as adult plants (Fig. 4), whereas deg15-1 and pxm16 seedlings and adult plants resembled wild type.

We examined mutants defective in two other putative peroxisomal proteases (At2g18080 and At2g35615, Table I), but did not find any differences from wild type in IBA resistance or sucrose dependence assays (data not shown). The only putative peroxisomal protease with a major peroxisome-targeting signal for which we did not identify a T-DNA insertion was the ovarian tumor (OTU)-like cysteine protease (At3g57810; Table I). At3g57810 is most highly expressed in flowers (Winter et al., 2007), which might explain the lack of seedling phenotypes and the absence of this protein from peroxisomal proteome studies, which have not sampled flowers (Fukao et al., 2002; Reumann et al., 2007; Arai et al., 2008; Eubel et al., 2008; Reumann et al., 2009).

lon2 Defects Are Enhanced by Disruption of DEG15

To assess genetic interactions among the peroxisomal proteases, we isolated lon2 deg15 and lon2 pxm16 double mutants. The deg15 mutant enhanced several lon2 defects. lon2-1 deg15-1 and lon2-2 deg15-1 double mutant seedlings (Fig. 2D and 3B) and adult plants (Fig. 4) were small and exhibited significantly enhanced sucrose dependence both in the dark and in the light (Fig. 2). Similarly, the lon2-1 deg15-1 and lon2-2 deg15-1 double mutant seedlings were more resistant to the inhibitory effect of IBA on root elongation than the single mutants (Fig. 3A). The lon2 mutants were completely insensitive to the tested IBA concentration in the lateral root promotion assay; lon2 deg15 double mutants resembled lon2 single mutants in this assay (Fig. 3C). In contrast, the pxm16 double mutants did not appear to enhance lon2 mutant defects; lon2 pxm16 double mutants resembled lon2 single mutants in sucrose dependence (Fig. 2), IBA resistance (Fig. 3), and adult size (Fig. 4).

LON2, DEG15, and PXM16 Are Not Necessary for Degradation of Glyoxylate Cycle Enzymes

The physiological defects of the lon2 mutants suggested that LON2 was required for peroxisomal function. Peroxisomal proteases might function to degrade damaged or obsolete peroxisomal proteins, such as the glyoxylate cycle enzymes malate synthase (MLS) and
isocitrate lyase (ICL), which are synthesized early in Arabidopsis postgerminative growth and then degraded after the onset of photoautotrophic growth (Cornah et al., 2004; Lingard et al., 2009). To determine whether LON2, DEG15, or PXM16 are necessary for the degradation of glyoxylate cycle enzymes during seedling development, we analyzed MLS and ICL stability in mutant seedlings. We found that MLS (Fig. 5) and ICL (Supplemental Fig. 1A) disappeared at similar rates in wild-type, lon2-1, lon2-2, and deg15-1 seedlings. Moreover, ICL disappeared at wild-type rates in pxm16-1, pxm16-2, and in mutants defective in two other putative peroxisomal proteases (At2g18080 and At2g35615; Supplemental Fig. 1, B and C). As in the single mutants, MLS was degraded with approximately wild-type kinetics in the lon2 deg15 and lon2 pxm16 double mutants (Fig. 6). We concluded that LON2, DEG15, and PXM16 are not required for degradation of glyoxylate cycle enzymes during seedling development, consistent with recent data suggesting that ICL and MLS may be removed from the peroxisome for degradation in the cytosol (Lingard et al., 2009).

LON2 Is Necessary for Thiolase Accumulation and PTS2 Protein Processing

DEG15 is a PTS2-processing protease (Helm et al., 2007; Schuhmann et al., 2008), and we confirmed the previously reported deg15-1 defect in cleaving the PTS2-containing N-terminal region of thiolase (Fig. 5C, Schuhmann et al., 2008). Thiolase abundance peaked in 3- to 4-day-old wild-type seedlings before declining to a lower basal level in older seedlings (Fig. 5) (Germain et al., 2001; Lingard et al., 2009). Unprocessed thiolase in deg15-1 accumulated similarly to processed thiolase in wild type (Fig. 5C), suggesting that PTS2 removal does not appreciably affect peroxisomal thiolase stability. pxm16-1 and pxm16-2 displayed wild-type thiolase processing and accumulation (Supplemental Fig. 1B). In both lon2-1 and lon2-2, we observed processed thiolase in young seedlings, indicating that LON2 is not required for PTS2 processing. However, thiolase levels were reduced in 5- to 8-day-old lon2 mutants compared to wild type (Fig. 5, A and B). We concluded that LON2 was necessary for normal thiolase accumulation during seedling development.

To determine whether the reduced thiolase accumulation in lon2 required PTS2 removal, we examined thiolase levels in lon2 deg15 double mutants. We found that, as in deg15-1, thiolase was wholly unprocessed in lon2-1 deg15-1 and lon2-2 deg15-1 and that the unprocessed thiolase appeared to be destabilized, as in lon2 (Fig. 6, A and B). Thus the apparent thiolase instability in lon2 did not require PTS2 removal by DEG15. In the lon2 pxm16 double mutants, thiolase displayed similar accumulation and processing as in the lon2 single mutants (Fig. 6, C and D), consistent with the lack of enhancement of lon2 physiological phenotypes by the pxm16 mutants (Fig. 2-4).
We also examined processing and accumulation of a second PTS2-containing protein, peroxisomal malate dehydrogenase (PMDH), in the protease mutants. PMDH accumulated in a pattern reciprocal to thiolase during seedling development, with relatively low levels immediately following germination that increased as seedlings matured (Fig. 5). As previously reported (Helm et al., 2007; Schuhmann et al., 2008), PMDH was largely unprocessed in the deg15-1 mutant (Fig. 5C). In lon2-1 and lon2-2, the developmental increase in PMDH levels was accompanied by the appearance of unprocessed PMDH in 5- to 6-day-old seedling (Fig. 5, A and B). Thus, whereas lon2 seedlings processed and accumulated the PTS2 proteins thiolase and PMDH normally for the first few days after germination, defects in accumulation (thiolase) or PTS2 removal (PMDH) became apparent with age in mutant seedlings.

PTS2-GFP Is Partially Mislocalized in lon2-2 Mutants

Because blocking PTS2 processing did not restore thiolase accumulation in the lon2 mutants, we tested whether the apparent thiolase instability and PMDH processing defects might reflect peroxisomal import defects. We examined a 35S-PTS2-GFP reporter (Woodward and Bartel, 2005) in wild type, lon2-2, and pex5-10, a mutant with matrix protein import defects (Zolman et al., 2005; Lingard et al., 2009). 35S-PTS2-GFP includes the first 147 base pairs of PED1, encoding the PTS2 targeting signal and cleavage site, fused to the 5’ end of GFP (Woodward and Bartel, 2005); hence, PTS2-GFP has the same targeting signal and processing site as PED1, the predominant seedling thiolase isoform (Germain et al., 2001). In wild-type seedlings, PTS2-GFP was present and fully processed in 3- to 7-day-old seedlings, whereas in pex5-10, PTS2-GFP, like thiolase and PMDH, was almost completely unprocessed (Fig. 7A). In lon2-2, PTS2-GFP processing resembled PMDH – fully processed in 3-day-old seedlings but only partially processed in 5- and 7-day-old seedlings (Fig. 7A).

To determine the subcellular localization of PTS2-GFP and explore the apparent correlation between PTS2-GFP import defects and cell maturity, we analyzed PTS2-GFP via confocal fluorescence microscopy in wild-type, lon2-2, and pex5-10 root cells. In wild-type roots, PTS2-GFP displayed punctate fluorescence typical of peroxisomal localization in both root tips (Fig. 7C) and in maturing cells several mm above the tip (Fig. 7B), whereas in pex5-10, PTS2-GFP appeared to be partially cytosolic and peroxisomal in both root tip and maturation zone cells (Fig. 7, F and G). In lon2-2 root tip cells, PTS2-GFP appeared to be predominantly peroxisomal (Fig. 7E). However, PTS2-GFP displayed only partial peroxisomal localization in the lon2-2 vasculature, and PTS2-GFP appeared to be predominantly cytosolic in epidermis and cortex cells of the root maturation zone (Fig. 7D).
LON2 Facilitates Peroxisome Matrix Protein Import

The defects in PMDH and PTS2-GFP processing and PTS2-GFP localization that we observed in older lon2 seedling extracts (Fig. 5 and 7A) and differentiated root cells (Fig. 7D) suggested that LON2 becomes necessary for peroxisomal import of matrix proteins as seedlings (or cells) mature. To determine whether LON2 also was necessary for import of native peroxisomal proteins, we immunolabeled cotyledon cells with antibodies to peroxisomal catalase (CAT), the PTS1 proteins MLS and hydroxypyruvate reductase (HPR), the PTS2 proteins thiolase and PMDH, and the membrane peroxin PEX14 (Fig. 8A). Cotyledons from 4- and 8-day-old seedlings were selected for labeling because Arabidopsis cotyledon cells do not divide during this period (Mansfield and Briarty, 1996), allowing observation of maturation-specific defects, and because MLS (Charlton et al., 2005) and HPR (Timm et al., 2008) are predominantly expressed in aerial tissues. Moreover, PMDH is not abundant in root tissue; the small amount of immunoreactivity observed in root tissue was also present in the pmdh1 pmdh2 double mutant and may result from a protein that cross-reacts with the PMDH antibody (Fig. 8B).

In cotyledons from both 4- and 8-day-old wild-type seedlings, we found the peroxisomal matrix and membrane proteins in punctate structures typical of peroxisomes, whereas all but the membrane protein PEX14 appeared to be largely cytosolic in the pex5-10 receptor mutant (Fig. 8A). In 4-day-old lon2-1 and lon2-2 seedlings, CAT, MLS, and thiolase appeared to be peroxisomal, whereas PMDH showed both peroxisomal and cytosolic labeling. The peroxisomal localization of thiolase and the dual localization of PMDH detected by immunolocalization at day 4 (Fig. 8A) were consistent with the presence of only processed thiolase but both processed and unprocessed PMDH detected by immunoblotting at day 4 (Fig. 8B). In 8-day-old seedlings, all of the monitored peroxisome matrix proteins (CAT2, HPR, and PMDH) appeared to be largely cytosolic in the lon2 mutants, although we observed occasional punctate labeling (Fig. 8A), consistent with the possibility that some peroxisomal import was still occurring and suggested by the detection of not only unprocessed but also processed PMDH in lon2 seedling extracts (Fig. 8B). Immunolabeling with anti-PEX14 antibodies revealed the presence of peroxisomes in wild type, lon2-1, lon2-2, and pex5-10 (Fig. 8A), indicating that the mutant defects likely resulted from impaired import of peroxisomal matrix proteins, rather than impaired peroxisome formation. We concluded that the lon2 mutant import defect extends beyond PTS2 proteins, such as PMDH and PTS2-GFP, and includes a variety of matrix proteins, including at least one PTS1 protein (HPR) as well as catalase, which has an atypical PTS (Kamigaki et al., 2003).
DISCUSSION

Proteases function to degrade entire proteins and to process proproteins, and thus can remove damaged or obsolete proteins and regulate metabolic and signaling pathways (Schaller, 2004). In this work, we demonstrated that the peroxisomal LON2 protease is needed for importing PTS1 and PTS2 proteins into the peroxisome matrix of maturing cotyledon and root cells of Arabidopsis seedlings.

One peroxisome-associated proteolytic process during Arabidopsis development is the degradation of the glyoxylate cycle enzymes ICL and MLS a few days after germination (Lingard et al., 2009). The five mutants that we identified in putative peroxisomal proteases (Table I) displayed wild-type degradation of these glyoxylate cycle enzymes (Fig. 5 and Supplemental Fig. 1). Moreover, combining lon2 alleles with deg15-1 or pxm16 alleles did not result in MLS stabilization (Fig. 6), suggesting that these proteins do not function redundantly to degrade obsolete glyoxylate cycle enzymes. Because LON2, DEG15, and PXM16 are the only predicted Arabidopsis proteases that have been detected in peroxisomes (Reumann et al., 2004; Helm et al., 2007; Ostersetzer et al., 2007; Eubel et al., 2008; Schuhmann et al., 2008; Reumann et al., 2009), and none of the corresponding mutants had defects in glyoxylate cycle enzyme turnover, it may be that these proteases are not responsible for bulk degradation of damaged or obsolete proteins. Indeed, recent data suggest that peroxisome-associated degradation (PexAD) of damaged or obsolete matrix proteins may require the assistance of PEX4 and PEX6 and export to the cytosol (Lingard et al., 2009). Our failure to detect matrix protein degradation defects in lon2 or pxm16 mutants indicates that the substrates of these proteases remain to be identified, and is consistent with the possibility that LON2 and PXM16 serve regulatory functions in peroxisomes.

Roles of DEG15 and PXM16 in Peroxisome Biogenesis

Our mutant analysis confirmed previous findings of a role for DEG15 in PTS2 processing (Helm et al., 2007; Schuhmann et al., 2008). Although the deg15-1 mutant is severely compromised in PTS2 removal from thiolase and PMDH, deg15-1 displayed at most mild peroxisome-deficient phenotypes (Figs. 2 and 3). Because the deg15 seedling phenotypes are much less severe than those of mutants lacking functional PTS2 proteins such as the PED1 thiolase (Figs. 2 and 3; Hayashi et al., 1998; Germain et al., 2001) or PMDH1 and PMDH2 (Pracharoenwattana et al., 2007), PTS2 removal does not appear to be necessary for stability or function of essential matrix proteins. Indeed, PTS2 signals are not removed from yeast peroxisomal proteins (reviewed in Olsen, 1998). However, the deg15 mutant enhancement of lon2 growth defects (Fig. 4) and sucrose dependence (Fig. 2) suggests that at least one of the
PTS2 proteins needed for β-oxidation functions more effectively following PTS2 removal, at least in the context of lon2 mutations.

The pxm16 mutants showed no aberrant phenotypes and did not enhance lon2 defects under any of the examined conditions (Fig. 2-4). By contrast, other mutants with undetectable defects in peroxisome biogenesis can dramatically enhance moderate or severe defects in other mutants. For example, the pex22-1 mutant has no detectable peroxisome-defective phenotype but dramatically enhances pex4-1 mutant defects (Zolman et al., 2005), and the slight sucrose dependence of lon2-1 and lon2-2 is markedly enhanced in lon2 deg15 double mutants (Fig. 2). The failure of pxm16 mutants to enhance lon2 mutant phenotypes suggests that PXM16 is not necessary for peroxisome biogenesis, IBA metabolism, or fatty acid β-oxidation. Given the role of PXM16 homologs in insulin degradation and wound response (Strassner et al., 2002; Schaller, 2004), it is possible that PXM16 functions in aspects of peroxisome metabolism not assayed here, such as pathogen or wound responses.

Roles of LON2 in Peroxisome Biogenesis

In contrast to deg15 and pxm16, the Arabidopsis lon2 mutants displayed severe defects in certain facets of peroxisome function and were notably smaller than wild type following growth in soil (Fig. 4). Although the lon2 alleles displayed only minor defects in seedling growth without sucrose supplementation (Fig. 2) and primary root elongation inhibition by IBA (Fig. 3, A and B), lon2 mutants were severely impaired in IBA-stimulated lateral root formation (Fig. 3C). In this regard, the lon2 mutant phenotype was unique compared to other peroxisome-defective mutants. Many mutants, like pex6-1 and pex5-10, show severe defects in both IBA responsive root elongation inhibition and lateral root promotion (Zolman et al., 2000; Zolman and Bartel, 2004; Zolman et al., 2005). However, previously characterized IBA-response mutants with mild defects in primary root response to IBA generally show only partial defects in lateral root induction. For example, the pex7-1 peroxin mutant displays moderately IBA resistant primary root elongation and partial defects in IBA-responsive lateral root promotion (Woodward and Bartel, 2005), and single mutants defective in three of the six Arabidopsis acyl-CoA oxidase genes display moderate IBA resistance in root elongation but no apparent defect in IBA-responsive lateral root promotion (Adham et al., 2005).

The lateral root assay, in which 4-day-old seedlings are transferred to IBA-containing medium for four additional days, may be particularly sensitive to developmentally delayed defects in peroxisome metabolism. The lon2 mutants displayed matrix protein import defects that appeared to correlate with cell age; differentiated root cells imported PTS2-GFP into peroxisomes much less efficiently than recently divided cells near the root tip (Fig. 7, D and E). Similarly, several PTS1 and PTS2 proteins were predominantly peroxisomal in 4-day-old lon2
cotyledon cells but largely cytosolic in 8-day-old cotyledon cells (Fig. 8A). These localization defects were accompanied by accumulation of both processed and unprocessed PMDH and PTS2-GFP in older lon2-2 seedlings (Fig. 5 and 7A). Although the T-DNA insertions that we identified in LON2 are near the C terminus and may not completely disrupt LON2 function, the lon2 protein products would lack the C-terminal PTS1 (Fig. 1A) and would be predicted to remain cytosolic. If present, it seems unlikely that cytosolic lon2 protein is responsible for the accelerated thiolase degradation we observed in lon2-1 and lon2-2, as thiolase stability in pex5-10 resembles wild type (Fig. 7A) even though thiolase (and presumably LON2) is largely cytosolic in pex5-10 (Fig. 8A). Moreover, we cannot eliminate the possibility that some lon2 protein enters the peroxisome, either through a cryptic PTS1 provided by the T-DNA border or by association with an interacting peroxisome-bound protein. The future characterization of demonstrated lon2 null alleles and LON2 antibodies would aid in resolving these ambiguities.

The H. polymorpha Lon isoform Pln appears to degrade certain peroxisome-targeted, non-native proteins (Aksam et al., 2007). Although Arabidopsis lon2 mutants did not display notably reduced degradation rates of the matrix proteins that we examined (Fig. 5 and 6; Supplemental Fig. 1), it is possible that Arabidopsis LON2 degrades one or more peroxisomal proteins that we did not examine. In lon2, the absence of this hypothetical degradation might block further matrix protein import, either because LON2 targets a regulatory protein, or because bulk undegraded protein in the peroxisome impairs further import.

Plant peroxisomal LON isoforms are more similar to chordate peroxisomal LON isoforms than either are to their yeast relatives (Fig. 1C). Moreover, phylogenetic analysis suggests that a subset of yeasts that includes H. polymorpha may have acquired a peroxisomal LON isoform in an evolutionary event distinct from the event in which the peroxisomal LON now found in plants and animals was obtained (Fig. 1C), consistent with the possibility that peroxisomal LON may function differently in these lineages. Arabidopsis lon2 mutants displayed striking matrix protein import defects (Fig. 7 and 8). Similarly, a mammalian cell line expressing a dominant negative pLon variant missorts catalase to the cytosol (Omi et al., 2008). Rather than acting as a protease, Arabidopsis LON2 may assist in matrix protein import as a chaperone, perhaps aiding in dissociation of the PEX5 and PEX7 receptors following import, or in dissociation of receptors and cargo, if such dissociations are necessary for receptor recycling. These models are consistent with the delayed onset of lon2 peroxisome-defective phenotypes. It is intriguing that certain eukaryotes, including Drosophila and C. elegans, appear to lack peroxisomal LON isoforms (Fig. 1C). C. elegans also lacks PEX7 (Gurvitz et al., 2000; Motley et al., 2000) and would not need a mechanism for PEX5-PEX7 dissociation, for example. Further examination of peroxisomal Lon function in a variety of eukaryotes, as well as the identification of LON2 substrates, will assist in distinguishing among these possibilities.
MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild type and mutants were in the *Arabidopsis thaliana* Col-0 accession. Seeds were surface sterilized, stratified in 0.1% [w/v] agar at 4 ºC for the indicated times, and germinated on nutrient media supplemented with sucrose and hormones as previously described (Zolman and Bartel, 2004). In auxin response assays, seedlings were grown in continuous light filtered through yellow long-pass filters to slow photochemical breakdown of auxin (Stasinopoulos and Hangarter, 1990).

Wild type (**35S-PTS2-GFP**) (Woodward and Bartel, 2005), *pex5-10* (Zolman et al., 2005), *pmdh1 pmdh2* (Pracharoenwattana et al., 2007), *mls-3* (Lingard et al., 2009), and *pex6-1* (Zolman and Bartel, 2004) were described previously. *lon2-1* (SALK_128438), *lon2-2* (SALK_043857), *pxm16-1* (SALK_019128), *pxm16-2* (SALK_023917), and *deg15-1* (SALK_007184) were from the Salk Institute sequence-indexed insertion collection (Alonso et al., 2003). *lon2-2* was backcrossed to Col-0 either once (Fig. 3C, 4-8) or twice (Fig. 2, 3A, and 3B). The sites of T-DNA insertions were verified by sequencing junctional PCR amplicons. *pex5-10* (**35S-PTS2-GFP**), *lon2-2* (**35S-PTS2-GFP**), and the protease double mutants were isolated by crossing and following mutations and transgenes via PCR (Supplemental Table I) in subsequent generations. We isolated *ped1-96* from an ethyl methanesulfonate-mutagenized Col-0 population as a sucrose-dependent and IBA-resistant seedling; the mutant harbors a C-to-T mutation in *PED1/KAT2* (*At2g33150*) that destroys a *Hin*P1 restriction enzyme site and replaces glutamine 190 with a stop codon (Supplemental Table I and Fig. 1B). *deg15-1* and *ped1-96* were backcrossed to Col-0 once prior to analysis.

Immunoblot Analysis

Cotyledons (Lingard et al., 2009) and whole seedlings (Zolman and Bartel, 2004) were prepared as previously described for western blot analysis. Immunoblotting was conducted as previously described (Lingard et al., 2009). Primary antibodies were diluted as follows: 1:25,000 α-MLS (Olsen et al., 1993), 1:2,500 α-thiolase (Lingard et al., 2009), 1:2,000 α-PMDH2 (Pracharoenwattana et al., 2007), 1:500 α-GFP (632377; Clontech, Mountain View, CA), and 1:8,000 α-HSC70 (SPA-817; StressGen Biotechnologies, Victoria, Canada).

Microscopy

Samples for immunofluorescence microscopy were prepared as described previously (Lingard et al., 2009), except that 8-day-old cotyledon cell walls were permeabilized in 1.5% driselase (Sigma) for 1 hour. Cotyledons were labeled with rabbit 1:2,000 α-CAT (Kunce et al.,
1988), 1:24,000 α-MLS (Olsen et al., 1993), 1:2,000 α-ICL (Maeshima et al., 1988), 1:2,000 (4-day-old-seedlings) or 1:1,500 (8-day-old-seedlings) α-PMDH2 (Pracharoenwattana et al., 2007), 1:2,500 α-PED1 (Lingard et al., 2009), 1:1,000 α-HPR (Kleczkowski and Randall, 1988), and 1:12,500 α-PEX14 (prepared from rabbits immunized with an E. coli-produced fusion protein including the N-terminal 243 amino acids of Arabidopsis PEX14/At5g62810) for 4 h at 37°C. The secondary antibody was 1:1,500 goat α-rabbit Alexa 594 (Invitrogen).

A Zeiss Axioplan 2 (Thornwood, NY) fluorescence compound microscope equipped with narrow band GFP filters (Chroma, Rockingham, VT) and a 40× lens was used for imaging immunolabeled tissues. Images were acquired with a CoolSNAP HQ camera (Photometrics, Tuscon, AZ) using MetaMorph 7 imaging software (Molecular Devices Corporation, Sunnyvale, CA). Confocal images of GFP-expressing tissues were acquired using a Zeiss LSM 510 confocal microscope (excitation 488 nm, emission 500-550 nm) equipped with a 20× lens. Images were cropped using NIH ImageJ and adjusted for brightness in Adobe Photoshop CS (San Jose, CA).

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FIGURE LEGENDS

Figure 1. Diagram of LON2 protein domains, gene models for LON2, DEG15, PXM16, PED1, PEX5, and PEX6, and phylogenetic relationships of LON family members. (A) Organization of the 888-amino acid LON2 protein. Locations of the N-terminal domain conserved among Lon proteins, predicted ATP-binding Walker A and B domains (filled circles), active site serine (S) and lysine (K) residues (asterisks), and the C-terminal serine-lysine-leucine (SKL) peroxisomal targeting signal (PTS1) are shown (Lee and Suzuki, 2008). (B) Gene models for LON2, DEG15, PXM16, PED1, PEX5, and PEX6 and locations of T-DNA insertions (triangles) or missense alleles used in this study (arrows). Exons are depicted by black boxes, introns by black lines, and untranslated regions by grey lines. (C) Phylogenetic relationships among LON homologs. Sequences were aligned using MegAlign (DNAStar) and the ClustalW method. The PAUP 4.0b10 program (Swofford, 2001) was used to generate an unrooted phylogram from a trimmed alignment corresponding to Arabidopsis LON2 residues 400-888 (from the beginning of the ATPase domain to the end of the protein). The bootstrap method was performed for 500 replicates with distance as the optimality criterion. Bootstrap values are indicated at the nodes. Predicted peroxisomal proteins have C-terminal PTS1 signals in parentheses and are in light gray ovals. Proteins in the darker gray oval have N-terminal extensions and include mitochondrial and chloroplastic proteins. Sequence identifiers are listed in Supplemental Table II.

Figure 2. The weak sucrose dependence of lon2 mutants is enhanced when combined with deg15-1. (A, B) Sucrose-dependent hypocotyl elongation in the dark. Seedlings were grown for 36 h in light and 4.5 days in darkness on media with or without 0.5% sucrose. (C, D) Sucrose dependent root elongation in the light. Seedlings were grown for 7 days under white light on media with or without 0.5% sucrose. Seeds were stratified for 1 day prior to plating. In panels A and C, error bars represent standard deviations of the means (n ≥ 12), numbers above light gray bars indicate the mean percentages of elongation on media without sucrose, and asterisks denote no sucrose values significantly different than sucrose values (Student’s t-test, P < 0.001). Panels B and D show three seedlings representing the range of observed phenotypes.

Figure 3. The IBA resistance of lon2 mutants is enhanced when combined with deg15-1. (A, B) IBA-resistant primary root elongation. After stratification for 1 day, seedlings were grown for 7 days under yellow light on sucrose-containing media supplemented with the indicated concentration of IBA. Asterisks denote values significantly different than wild type (Student’s t-test, P < 0.001). Three seedlings representing the range of observed phenotypes in panel A are shown in panel B. (C) IBA promotion of lateral root formation. After stratification for 3 days, seedlings were grown for 4 days on hormone-free media and then transferred to media.
containing the indicated hormone. After 4 additional days, lateral roots were counted and primary root lengths were measured. Error bars represent standard deviations of the means \((n \geq 12)\) (A and C).

**Figure 4.** *lon2* mutants display growth defects that are enhanced by *deg15-1*. Representative 28-day-old plants of the indicated genotypes are shown. Scale bar = 2 cm.

**Figure 5.** *lon2* mutants degrade MLS normally but have defects in PTS2-containing protein accumulation and processing. (A-C) Immunoblots of total protein extracted from wild-type and *lon2-1* (A), *lon2-2* (B), or *deg15-1* (C) cotyledons (12 per lane) from 3- to 8-day-old seedlings. Blots were probed with antibodies raised against the PTS1-containing protein MLS, the PTS2-containing proteins PED1 (thiolase) and PMDH2, and HSC70 as a loading control. The positions of molecular weight markers (in kDa) are indicated on the left; processed and unprocessed PTS2 proteins are marked by one or two asterisks, respectively.

**Figure 6.** *lon2 deg15* double mutants do not process PTS2-containing proteins and have thiolase accumulation defects. (A-D) Immunoblots of protein extracted from 3-, 5-, and 7-day-old wild type and *lon2* single and double mutants (7 seedlings per lane). Seeds were stratified for 6 days prior to plating. Blots were sequentially probed with anti-PED1 (thiolase), PMDH2, MLS, and HSC70 antibodies. The positions of molecular weight markers (in kDa) are indicated on the left; processed and unprocessed PTS2 proteins are marked by one or two asterisks, respectively.

**Figure 7.** *lon2-2* roots have tissue-specific defects in PTS2-GFP import. (A) Immunoblot of protein extracted from 3-, 5-, and 7-day-old wild-type (*35S-PTS2-GFP*), *lon2-2* (*35S-PTS2-GFP*), and *pex5-10* (*35S-PTS2-GFP*) seedlings (8 per lane). Seeds were stratified for 2 days prior to plating. Blots were probed with anti-PED1 (thiolase), PMDH2, GFP, and HSC70 antibodies. Positions of molecular weight markers (in kDa) are indicated on the left; processed and unprocessed PTS2 proteins are marked by one or two asterisks, respectively. (B-G) Root tip cells (C, E, G) and maturing cells several mm above the tip (B, D, F) of 6-day-old seedlings expressing PTS2-GFP were imaged via confocal microscopy. Micrographs are 11 µm thick individual optical sections. Scale bar = 100 µm.

**Figure 8.** *lon2* mutant cotyledon cells import some matrix proteins normally at 4 days old, but matrix protein import is impaired at 8 days. (A) Cotyledons from 4- and 8-day-old light-grown *Arabidopsis* seedlings were immunolabeled with antibodies raised against catalase (CAT), PTS2 proteins (PED1 and PMDH2), PTS1 proteins (MLS and HPR), and a membrane-bound peroxin.
Supplemental Figure 1. The glyoxylate enzyme isocitrate lyase is degraded with near wild-type kinetics in all identified putative peroxisomal protease mutants. (A) Immunoblots of protein extracts from wild-type (Col-0), deg15-1, lon2-1, and lon2-2 cotyledons (12 per lane) cut from 3- to 8-day-old light-grown seedlings. (B) Immunoblot of protein extracts from deg15-1, pxm16-1, pxm16-2, and lon2-2 cotyledons (12 per lane) from 3- and 6-day-old light-grown seedlings. (C) Immunoblot of protein extracts from cotyledons of mutants disrupted in putative peroxisomal proteases At2g18080 and At2g35615 from 3- and 6-day-old light-grown seedlings. Blots were probed with antibodies raised against isocitrate lyase (ICL), hydroxypyruvate reductase (HPR), PED1 (thiolase), and HSC70. Positions of molecular weight markers (in kDa) are indicated on the left. Processed and unprocessed thiolase are marked by one or two asterisks, respectively, and a filled circle marks the position of a protein that cross-reacts with the ICL antibody but is not found in the icl null mutant (Zolman et al., 2005).
| AGI identifier | Alias | Protein class | T-DNA insertion alleles | PTS Localization | Localization references |
|---------------|-------|---------------|-------------------------|------------------|------------------------|
| At1g28320     | DEG15 | PTS2-processing protease | SALK_007184 (deg15-1) | SKL> | GFP Proteomics Bioinformatics (Reumann et al., 2004; Helm et al., 2007; Eube et al., 2008; Schuhmann et al., 2008) |
| At2g41790     | PXM16 | Peptidase M16 family protein | SALK_019128 (pxm16-1), SALK_023917 (pxm16-2) | PKL> | Proteomics Bioinformatics (Reumann et al., 2004; Eubel et al., 2008; Reumann et al., 2009) |
| At5g47040     | LON2  | Lon protease homolog | SALK_128438 (lon2-1), SALK_043857 (lon2-2) | SKL> | GFP Proteomics Bioinformatics (Reumann et al., 2004; Ostersetzer et al., 2007; Eubel et al., 2008; Reumann et al., 2009) |
| At2g18080     |       | Serine-type peptidase | SALK_020628, SALK_102239 | SSI> | Bioinformatics (Reumann et al., 2004) |
| At2g35615     |       | Aspartyl protease | SALK_090795, SALK_036333 | ANL> | Bioinformatics (Reumann et al., 2004) |
| At3g57810     |       | OTU-like cysteine protease | | SKL> | Bioinformatics (Reumann et al., 2004) |
| At4g14570     |       | Acylaminoacyl-peptidase protein | | CKL> | Bioinformatics (Reumann et al., 2004; Shimaoka et al., 2004) |
| At4g20310     |       | Peptidase M50 family protein | | RMx5HL | Bioinformatics (Reumann et al., 2004) |
| At4g36195     |       | Serine carboxypeptidase S28 family | | SSM> | Bioinformatics (Reumann et al., 2004) |

aMajor PTS1 (Reumann, 2004).
bMinor PTS1 (Reumann, 2004).
cValidated PTS1 (Reumann et al., 2007).
dMinor PTS2 (Reumann, 2004).
