Differential Contribution of Two Peroxisomal Protein Receptors to the Maintenance of Peroxisomal Functions in Arabidopsis*

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Peroxisomes in higher plant cells are known to differentiate in function depending on the cell type. Because of the functional differentiation, plant peroxisomes are subdivided into several classes, such as glyoxysomes and leaf peroxisomes. These peroxisomal functions are maintained by import of newly synthesized proteins containing one of two peroxisomal targeting signals known as PTS1 and PTS2. These targeting signals are known to be recognized by the cytosolic receptors, Pex5p and Pex7p, respectively. To demonstrate the contribution of Pex5p and Pex7p to the maintenance of peroxisomal functions in plants, double-stranded RNA constructs were introduced into the genome of Arabidopsis thaliana. Expression of the PEX5 and PEX7 genes was efficiently reduced by the double-stranded RNA-mediated interference in the transgenic Arabidopsis. The Pex5p-deficient Arabidopsis showed reduced activities for both glyoxysomal and leaf peroxisomal functions. An identical phenotype was observed in a transgenic Arabidopsis overexpressing functionally defective Pex5p. In contrast, the Pex7p-deficient Arabidopsis showed reduced activity for glyoxysomal function but not for leaf peroxisomal function. Analyses of peroxisomal protein import in the transgenic Arabidopsis revealed that Pex5p was involved in import of both PTS1-containing proteins and PTS2-containing proteins, whereas Pex7p contributed to the import of only PTS2-containing proteins. Overall, the results indicated that Pex5p and Pex7p play different roles in the maintenance of glyoxysomal and leaf peroxisomal functions in plants.

Peroxisomal enzymes are synthesized in the cytosol and function after their post-translational transport into peroxisomes. Most of the plant peroxisomal enzymes have been shown to contain one of two peroxisomal targeting signals within their amino acid sequences (1, 2). One type of targeting signal, called PTS1 (peroxisome targeting signal), is a unique tripeptide sequence found in the carboxyl terminus of the proteins (3, 4). In higher plant cells, the permissible combinations of tripeptide sequence for PTS1 are (C/A/S/P)(K/R)(I/L/M) (5). Another type of targeting signal, PTS2, is involved in a cleavable amino-terminal presequence (6). The amino-terminal presequences contain a consensus sequence (X/L/Q/I/X3(H/L/I/F) where X indicates any amino acid) (7, 8). PTS2 is processed to form the mature protein after its transport into peroxisomes. Peroxisomes in higher plant cells have been shown to differentiate into at least three different classes, namely glyoxysomes, leaf peroxisomes, and unspecialized peroxisomes (9). Each organelle contains a unique set of enzymes that provides special functions in various organs in higher plants. Glyoxysomes are present in cells of storage organs, such as endosperms and cotyledons during post-germinative growth of oilseed plants, as well as in senescent organs (10). They contain enzymes for fatty acid β-oxidation and the glyoxylate cycle and play a pivotal role in the conversion of seed-reserved lipid into sucrose. It has been suggested that fatty acids produced from the lipid are exclusively degraded in glyoxysomes (i.e. not in mitochondria) during germination and post-germinative growth in plants (11). By contrast, leaf peroxisomes are widely found in cells of photosynthetic organs. It has been shown that some enzymes responsible for photosynthesis are localized in leaf peroxisomes even though the entire photosynthetic process involves a combination of enzymatic reactions that occur in chloroplasts, leaf peroxisomes, and mitochondria (12). We recently identified 256 candidates of peroxisomal genes encoding either PTS1-containing proteins or PTS2-containing proteins within the entire Arabidopsis genome (13). Of these, the functions of only 29 gene products have been identified. By using a custom-made microarray, we extensively examined the expression of all these genes in various organs. Statistical analyses of the expression profiles revealed that peroxisomal genes could be classified into five groups. From the results, we suggested that plant peroxisomes can be subdivided into at least five different classes, namely glyoxysomes, cotyledonary peroxisomes, leaf peroxisomes, root peroxisomes, and unspecialized peroxisomes. However, the mechanism to maintain the functional differentiation of plant peroxisomes is still unknown.

Recent analyses of peroxisome-deficient mutants allowed the identification of over 25 PEX genes and their products, peroxins (14), from various organisms. Fifteen PEX gene orthologues exist in the Arabidopsis genome (15), and Arabidopsis mutants with defective PEX2, PEX5, PEX6, PEX10, PEX14, and PEX16 were reported (16–22). Of these, Pex14p is a peroxisomal membrane protein encoded by PED2 (Arabidopsis orthologue of PEX14) and is believed to contribute to both PTS1- and PTS2-dependent protein targeting pathways, because the Arabidopsis ped2 mutant has defects in intracellular transport of both PTS1- and PTS2-containing proteins (16). In contrast, Pex5p (PEX5 product) and Pex7p (PEX7 product) function as cytosolic receptors for

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‡ The abbreviations used are: Pex5p, peroxisome targeting signal 1; Pex7p, peroxisome targeting signal 2; 2,4-D, 2,4-dichlorophenoxoisobutyric acid; GFP, green fluorescent protein; MES, 2-(N-morpholino)ethanesulfonic acid; Ti, tumor-inducing; Pa, pascal.
PT51- and PT52-containing proteins, respectively (23). Here we report on transgenic plants that had defects in Pe5p and Pe7p. Based upon the phenotypes of the transgenic plants, we show evidence that Pe5p and Pe7p contributed differently to the maintenance of the peroxisomal functions in plants.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—Arabidopsis thaliana ecotype Columbia was used as the wild-type plant. Construction of transgenic plants expressing GFP-PTS1 and PT52-GFP and characterization of the ped2 mutant were described previously (16, 24). All seeds were surface-sterilized and grown on growth medium (2.3 mg/ml Murashige and Skoog salts (Wako, Osaka, Japan), 1% sucrose, 100 μg/ml mycinoisol, 1 μg/ml thiamine-HCl, 0.5 μg/ml pyridoxine, 0.5 μg/ml nicotinic acid, 0.5 mg/ml MES-KOH, pH 5.7, and 0.8% agar) with or without antibiotic(s). In some experiments, 0.2 μg/ml of 2,4-dichlorophenoxybutyric acid was added, or sucrose was removed from the growth medium. Seedlings were then transferred and grown on a 1:1 mixture of perlite and vermiculite under a 16-h-light (100 μE m⁻² s⁻¹)-8-h-dark light cycle at 22 °C in a normal atmosphere or in an atmosphere containing 1000 Pa CO₂.

Construction of Artificial Genes—Isolation of PT5X and PT5X cDNAs was reported previously (23). For making the pex5i and pex7i constructs, the gene-specific sequences in the sense orientation were amplified by PCR to introduce SpeI and ScaI sites at each end, whereas BamHI and Xhol sites were introduced into the same sequence of the antisense orientation. The sense and antisense DNA fragments were inserted between SpeI-ScaI and XhoI-BamHI sites of the same plasmid as a linker. The XhoI-SacI DNA fragments were removed from the plasmids and ligated into the XhoI-BamHI site of the same plasmid as a linker. The XhoI-SacI DNA fragments were inserted between SpeI-SacI and XhoI-BamHI sites of pBluescript KS (Stratagene, La Jolla, CA), respectively. A BamHI-BgII fragment encoding a bar gene from the pARK22 plasmid was also inserted at a BamHI site of the same plasmid as a linker. The XhoI-SacI DNA fragments were removed from the plasmids and ligated into the XhoI-SacI site of pBI121-Hm linker. The pBI121-Hm linker was constructed from a Ti vector, pBI121, by replacing the GUS gene with a linker sequence containing XbaI, Xhol, Smal, SpeI, KpnI, and ScaI sites. Also, using a hygromycin-resistant gene as a selectable marker in plants. To make the PEX5Δ7 construct, a DNA fragment encoding the Met1–Tyr657 region of Pex5p (open box) was amplified by PCR. It was inserted into a pDONR221 plasmid (Invitrogen) and then transferred into a Ti vector, pGWB2 (kindly gifted from Dr. Nakagawa), using BP and LR clonases according to the manufacturer's specifications (Invitrogen).

Agrabacterium-mediated Transformation—TI vectors carrying pex5i, pex7i, PEX5Δ7, and PEX7Δ7 were independently introduced into lethargy-le1 and kanamycin-resistant parental transgenic Arabidopsis plants expressing GFP-PTS1 and PT52-GFP (24) by vacuum infiltration (25) using Agrobacterium tumefaciens (strain C58C1RI(β)). Transformed Arabidopsis lines were selected on growth medium containing 50 μg/ml kanamycin and 25 μg/ml hygromycin.

Immunoblotting—Sample preparation and immunoblot analyses using antibodies against AtPex5p and AtPex7p were performed as previously reported (23). Crude extract was prepared from leaves of the 3-week-old T1 plant. Ten μg of the total protein was used for immunoblotting.

RESULTS

Generation of Transgenic Plants That Have a Defect in Pex5p or Pex7p—Double-stranded RNA interference was used to induce post-transcriptional gene silencing of PEX5 and PEX7. To induce double-stranded RNA interference in Arabidopsis, we constructed two artificial genes, pex5i and pex7i, that encode RNAs capable of double strand formation at gene-specific sequences. Transformation and selection were performed by a method reported previously (27). As shown in Fig. 1A, both constructs contain two identical sequences encoding a part of the corresponding protein in antisense and sense orientations and were linked together by a bar gene. In parallel, we constructed PEX5Δ7 overexpressing the Met1–Tyr657 region of Pex5p lacking 71 amino acid residues at the carboxyl terminus (Fig. 1A). This transgenic plant was constructed because we expected that the defective Pex5p protein would induce a dominant negative phenotype. These DNA fragments were inserted under the control of a constitutive 35S promoter from cauliflower mosaic virus in a Ti vector containing a hygromycin-resistant gene as a selectable marker in plants, and then integrated into the genomes of two kanamycin-resistant transgenic plants, AtGFP-PTS1 and AtPTS2-GFP, by Agrobacterium-mediated transformation. Construction of these parental lines has been described previously elsewhere (24).

Measurements of Maximum Quantum Yield of Photosystem II (Fv / Fm)—To reduce the effect of photo-inhibition, plants were grown for 4 weeks in an atmosphere containing 1000 Pa CO₂ under low light (50 μE m⁻² s⁻¹) for 9 and 4 h in a normal atmosphere (36 Pa CO₂). At the end of each illumination period, plants were kept for 30 min in the dark. The ratio of variable fluorescence to maximum fluorescence (Fv / Fm) was automatically calculated from the results of the modulated chlorophyll fluorescence emission from the upper surface of dark-adapted leaves that was measured using a pulse amplitude modulation fluorometer, Mini-PAM (H. Walz, Effeltrich, Germany).

Detection of Fluorescence Derived from GFP-PTS1 and PT52-GFP—For analyzing root cells, T1 plants, grown 10 days on growth medium, were mounted under a coverslip. For analyzing leaf cells, leaves were removed from the 3-week-old plants were mounted under a coverslip. Fluorescent images of the specimens were captured using an LSM 510 laser-scanning confocal microscope with an argon laser and a fluorescein isothiocyanate filter set (emission 500–550 nm) (Carl Zeiss, Oberkochen, Germany).

Yeast Two-hybrid Analyses—Yeast two-hybrid analyses of PEX5Δ7 were performed according to protocols reported previously (23). A DNA fragment encoding PEX5Δ7 in a pDONR plasmid was transferred into a pGAD-GW plasmid. The yeast two-hybrid vector, pGAD-GW, was constructed from pGAD-C1 (26) by replacing the multilocining site with a gateway conversion cassette (Invitrogen). Construction of other vectors used in this study have been described elsewhere (23). Transformants were tested for growth on synthetic medium containing 50 mM 3-aminothiazole without histidine and were assayed for quantitative β-galactosidase activity.
The retransformation of pex5i, pex7i, and PE
X5Δ7 into the AtGFP-PTS1 and AtPTS2-GFP plants allowed us to determine the effects of these DNA constructs on import of the GFP-PTS1 and PTS2-GFP in vivo. The primary transformant was designated as T0 plants. T1 progeny showing both kanamycin and hygromycin resistance were designated as pex5i/GFP-PTS1, pex5i/PTS2-GFP, pex7i/GFP-PTS1, pex7i/PTS2-GFP, PEX5Δ7/GFP-PTS1, and PEX5Δ7/PTS2-GFP.

The effects of the transgenes in the T1 progeny were examined by immunoblot analysis. In both pex5i/GFP-PTS1 and pex5i/PTS2-GFP the amounts of Pex5p were greatly reduced, whereas no effect was observed for the amounts of Pex7p (Fig. 1B, pex5i (lane 1) and pex5i (lane 2)). Inversely, the amounts of Pex7p were greatly reduced, whereas no effect was observed for the amounts of Pex5p in both pex7i/GFP-PTS1 and pex7i/PTS2-GFP (Fig. 1B, pex7i (lane 1) and pex7i (lane 2)). PEX5Δ7/GFP-PTS1 and PEX5Δ7/PTS2-GFP contained an additional form of Pex5p whose molecular mass was well matched with the polypeptide encoded by PEX5Δ7, whereas no effect was observed for Pex7p (Fig. 1B, PEX5Δ7 (lane 1) and PEX5Δ7 (lane 2)). It is noteworthy that all of these samples contained similar amounts of ribulose-bisphosphate carboxylase/oxygenase (data not shown). All phenotypes described in this study were dominant.

Effects of 2,4-Dichlorophenoxacycetic Acid and Sucrose on Growth of Pex5i, Pex7i, and PEX5Δ7—We reported previously (28) that plants lacking glyoxysomal fatty acid β-oxidation become resistant to 2,4-dichlorophenoxacycetic acid (2,4-DB) and require sucrose during postgerminative growth. To analyze the contribution of Pex5p and Pex7p to glyoxysomal function, the effects of 2,4-DB and sucrose were examined using T2 progeny of the transgenic lines. When these T2 populations were grown on medium containing 2,4-DB, ~75% of the T2 seedlings had elongated roots, indicating resistance to 2,4-DB. The 2,4-DB-resistant phenotypes are shown in Fig. 2, upper panel. Because of genetic segregation of the transgene in the T2 population, the rest of the seedlings showed strong growth inhibition identical with their parental plants. In addition, ~75% of T2 seedlings required sucrose for post-germinative growth. These seedlings could not grow normally in the absence of sucrose (Fig. 2, middle panel). All seedlings that showed 2,4-DB resistance or required sucrose were resistant to both hygromycin and kanamycin (data not shown). These results indicated that introduction of the pex5i, PEX5Δ7, and pex7i constructs into the transgenic plants caused the defect in glyoxysomal function.

Activity of Photorespiration in pex5i, PEX5Δ7, and Pex7i—T2 progeny of pex5i/GFP-PTS1, PEX5Δ7/GFP-PTS1, and pex7i/PTS2-GFP, showing 2,4-DB resistance and cytosolic GFP fluorescence (see following description), were further cultivated in order to examine photorespiration in the leaf peroxisomes. When pex5i/GFP-PTS1 and PEX5Δ7/GFP-PTS1 were grown in a normal atmosphere (36 Pa CO₂), they had yellow-green leaves and showed a dwarf phenotype compared with wild-type plants (Fig. 3A, pex5i (air) and PEX5Δ7 (air)). This phenotype was recovered when the plants were grown under high CO₂ conditions (1000 Pa CO₂) (Fig. 3A, pex5i(CO₂) and PEX5Δ7(CO₂)). A similar phenomenon was observed in the ped2 mutant that has a defective PEX14 gene conferring reduced activity for photorespiration (16). In contrast, pex7i/PTS2-GFP grew normally even in the normal atmosphere (Fig. 3A, pex7i (air)). Based on these results, we assume that loss of Pex5p function reduces the activity of photorespiration in leaf peroxisomes, whereas loss of Pex7p does not.

We tested this hypothesis by measuring the maximum quantum yield of photosystem II, which can be estimated from the ratio of the variable fluorescence of dark-adapted chlorophyll a to the maximum fluorescence (Fo/Fm) (29–31). We compared Fo/Fm of dark-adapted leaves of the parental plants with pex5i/GFP-PTS1 plants and pex7i/PTS2-GFP plants (Fig. 3B).
They were initially grown for 3 weeks in an atmosphere containing high CO₂ (1000 Pa) under low light (50 μE m⁻² s⁻¹) to reduce the effects of photoinhibition. All plants grown in this condition showed normal growth and had similar Fv/Fm values (Fig. 3B, 0 h). These plants were then transferred to a normal atmosphere (36 Pa CO₂), where they were illuminated with a strong light (450 μE m⁻² s⁻¹). Under these conditions, ribulose-bisphosphate carboxylase/oxygenase acts as an oxygenase in addition to its carboxylase activity, which is necessary for CO₂ fixation in photosynthesis. Phosphoglycolate, a byproduct of the oxygenase reaction, is metabolized by photorespiration enzymes and finally returned to the Calvin-Benson cycle. Therefore, the Fv/Fm of the wild-type plants would not be expected to change after the strong illumination, and this is what was observed (Fig. 3B, GFP-PTS1(4 h) and PTS2-GFP(4 h)). In contrast, significant reduction of Fv/Fm was observed in the pex5i/GFP-PTS1 as well as the ped2 mutant, which has a defective PEX14 gene (Fig. 3B, pex5i/PTS1(4 h) and ped2(4 h)). Only a weak reduction of Fv/Fm was observed in pex7i/PTS2-GFP (Fig. 3B, pex7i/PTS2(4 h)). These results indicate that pex5i/GFP-PTS1 plants were unable to maintain sufficient activity of the Calvin-Benson cycle under low CO₂/strong light conditions because of the defect in photorespiration. The imbalance between the light reaction and the Calvin-Benson cycle might have been responsible for the observed reduction of the Fv/Fm. These results indicated that Pex5p, but not Pex7p, is necessary for the maintenance of the activity of photorespiration in leaf peroxisomes.

Intracellular Transport of Peroxisomal Proteins in the Absence of Pex5p—Subcellular localization of GFP fusion proteins, GFP-PTS1 and PTS2-GFP, in cells of pex5i/GFP-PTS1 and pex5i/PTS2-GFP was determined using a confocal laser-scanning microscope. Both leaf and root cells of pex5i/GFP-PTS1 showed green fluorescence only in the periphery of the cells (Fig. 4, upper panels) that coincided with the cytosol surrounding the central vacuoles. Identical fluorescent images were obtained in 19 independently isolated transformants for pex5i/GFP-PTS1 out of 20 T1 progenies showing hygromycin/kanamycin resistance. Cytosolic fluorescence was also obtained in leaf and root cells of 35 independent transformants for pex5i/PTS2-GFP out of the 42 T1 progenies (Fig. 4, middle panels). In contrast, cells of parental transgenic plants, AtpGFP-PTS1 and Pex7p/GFP-PTS1, showed punctated green fluorescence that coincided with peroxisomes (Fig. 4, lower panels). These data indicate that Pex5p mediates intracellular transport of both PTS1-containing and PTS2-containing proteins.

Effect of PEX5Δ7 Gene Product on Intracellular Transport of Peroxisomal Proteins—Fig. 6 shows subcellular localization of GFP-PTS1 and PTS2-GFP in leaf and root cells of PEX5Δ7/GFP-PTS1 and PEX5Δ7/PTS2-GFP transformants. Leaf and root cells of both PEX5Δ7/GFP-PTS1 and PEX5Δ7/PTS2-GFP had green fluorescence only in the cytosol. Identical fluorescent images were obtained in 5 transformants for PEX5Δ7/GFP-PTS1 and 6 for PEX5Δ7/PTS2-GFP out of 10 and 8 T1 progenies, respectively. These data indicate that the ability for intracellular transport of both PTS1-containing and PTS2-containing proteins was reduced when the PEX5Δ7 gene product was overexpressed in the cells.

Intracellular Transport of Peroxisomal Proteins in the Absence of Pex7p—Fig. 7 shows subcellular localization of GFP-PTS1 and PTS2-GFP in leaf and root cells of the pex7i/GFP-PTS1 and pex7i/PTS2-GFP. All 55 independent transformants for pex7i/GFP-PTS1 showed punctated fluorescence that was identical to their parental plants (Fig. 7, upper panels), although we confirmed that these plants contained reduced Pex7p.

AtPTS2-GFP, showed punctated green fluorescence that coincided with peroxisomes (Fig. 4, lower panels). These data indicate that Pex5p mediates intracellular transport of both PTS1-containing and PTS2-containing proteins.

Protein-Protein Interaction of PEX5Δ7 Gene Product—By employing a yeast two-hybrid system, co-immunoprecipitation assays, and pull-down assays, we demonstrated previously (23) that Arabidopsis Pex5p has the ability to bind not only to PTS1-containing proteins but also Pex7p and Pex14p in vitro (also see Fig. 5). Pex5p possesses a tetratricopeptide repeat motif at the carboxyl terminus (32, 33). The tetratricopeptide repeat motif consists of seven tandem repetitive consensus amino acid sequences that are necessary to interact with PTS1 (34, 35). The PEX5Δ7 gene product, Pex5Δ7p, is missing the last repeat in the tetratricopeptide repeat motif. Binding activities of Pex5Δ7p with PTS1 proteins Pex7p and Pex14p were examined by using yeast two-hybrid analysis according to a method reported previously (23). Each protein was fused to Gal4-AD or Gal4-AD and expressed in a yeast tester strain PJ69-4A (26). Protein-protein interactions were monitored by the Gal4-dependent transcriptional activation of HIS3 and β-galactosidase reporter genes (Fig. 5). No significant transcriptional activation of HIS3 and β-galactosidase reporter genes indicated that Pex5Δ7p could not bind with PTS1-containing protein. Binding activity between Pex5Δ7p and Pex7p, however, was identical to that between Pex5p and Pex7p, whereas Pex14p showed weaker binding activity to Pex5Δ7p than Pex5p.
and Pex5p, to the maintenance of peroxisomal functions in Pex7p-deficient plant (pex7i), and we have shown the differentiation but not for the maintenance of leaf peroxisomal function. Pex7p is necessary for the maintenance of glyoxysomal function, including higher plants (36). This silencing is induced by sequence-specific RNA degradation of double-stranded RNA. To analyze the functions of Pex5p and Pex7p in vivo, we induced gene silencing in Arabidopsis plants transformed with artificial genes encoding a hairpin RNA consisting of an inverted repeat of a target sequence. We previously reported protein-protein interaction among PEX5 and PEX7 genes (27, 37, 38). Here we showed that this method also was effective trigger of gene silencing in a number of organisms, i.e. the biosynthesis of plasmalogens, the α-oxidation of phytanic acid, and β-oxidation of very long chain fatty acid. These results indicated that Pex7p plays an important role in defining the distinct characteristics of peroxisomal function(s) in different multicellular organisms, such as higher plants and mammals.

Phenotypes of the Pex5p-deficient plant (pex5i) clearly indicated that Pex5p is necessary for the maintenance of both glyoxysomal and leaf peroxisomal functions. This conclusion was also supported by the dominant negative phenotype observed in PEX5Δ7, a transgenic Arabidopsis overexpressing functionally defective Pex5p, that had defective glyoxysomes and leaf peroxisomes. An Arabidopsis pex5 mutant has been identified previously (21) from progenies of ethyl methanesulphonate-mutagenized seeds. Although this mutant also had defective glyoxysomes, defects in leaf peroxisomal function were not observed. This may be because the missense mutation (Ser118 to Leu) may cause a leaky phenotype in the mutant.

Despite the advantage of RNA interference, significant numbers of the later generations lost their cytosolic fluorescence and regained fluorescence in their peroxisomes during growth and development (data not shown). One of the possible explanations for this is that the severe phenotypes might tend to release the gene silencing effects by an unknown influence of the Met1–Val230 region of Pex5p and the Tyr266–Ser317 region of Pex7p could bind to each other. Binding of nine WXX(W/F/Y) repeats in Pex5p with two domains (Ile65–Leu69 and Arg78–Arg97) of Pex14p was also seen, whereas no binding was observed between Pex5p and Pex14p. From these in vitro analyses, we suggested that the mechanism of peroxisomal protein targeting in higher plant cells is as follows. In the cytosol, Pex5p and Pex7p bind to each other to form a Pex5p-Pex7p receptor complex that catches both PTS1- and PTS2-containing proteins. The receptor-cargo complex captures peroxisomes by binding with Pex14p, a peroxisomal membrane protein, and with Pex5p, and the cargo is then transferred into the peroxisomal matrix.

This hypothesis is well matched to our present study showing peroxisomal protein import in the Pex5p- and Pex7-deficient plants. When Pex5p is missing, import of not only PTS1-containing proteins but also PTS2-containing proteins is abolished because of the loss of the Pex5p-Pex7p receptor complex (Fig. 4). Dependence of PTS2-containing protein import on Pex5p was also examined by overexpression of Pex5Δ7p in which binding activity with the cargo, PTS1-containing proteins, is completely lost (Fig. 6). In this case, excess amounts of Pex5Δ7p bind with Pex7p to form a disordered complex. Because of the weak binding activity of Pex5Δ7p to Pex14p and regulating functional differentiation of peroxisomes and defines temporally and spatially limited differentiation of peroxisomes, i.e. glyoxysomes found only in etiolated cotyledons during the early stage of postembryonic growth. It is noteworthy that the amount of Pex7p reduced in parallel with the functional differentiation of peroxisomes from glyoxysomes to leaf peroxisomes induced by illumination of the cotyledons (23). It has been shown that Rhizomelic chondrodysplasia punctata, a human autosomal recessive disorder, is caused by mutations in the PEX7 gene. In a recent study (39) of Pex7−/− mice, the model animals for this disorder indicated that symptoms of this disorder, such as cataracts, rhizomelia, and epiphyseal calcifications, may be induced by abnormalities of peroxisomal functions, i.e. the biosynthesis of plasmalogens, the α-oxidation of phytanic acid, and β-oxidation of very long chain fatty acid. We should emphasize that this is the first analysis of a human autosomal disorder, is caused by mutations in the PEX7 gene.
the loss of cargo, the disordered complex could not be identified by Pex14p, resulting in a disturbance of the import of not only PTS1- but also PTS2-containing proteins (Fig. 5). When Pex7p is missing, however, transport of only PTS2-containing proteins is abolished. Pex7p-deficient Arabidopsis showed normal activity for import of PTS1-containing proteins, because Pex5p might act as a receptor for PTS1-containing proteins and bind to Pex14p, without forming receptor complex with Pex7p (Fig. 7).

The contribution of Pex5p on the import of PTS2-containing proteins is species-dependent. Arabidopsis has only one PEX5 orthologue. Our extensive search for this transcript suggests that Arabidopsis may only produce a single form of Pex5p (15, 23). Therefore, all Pex5p expressed in Arabidopsis cells could mediate not only PTS1- but also PTS2-dependent protein targeting pathways. In mammalian cells, however, two isoforms of Pex5p (Pex5pL and Pex5pS) are produced by alternative splicing (40). Pex5pL, containing an additional 37 amino acids, mediates a PTS2-dependent protein targeting pathway, whereas Pex5pS does not (41, 42). In yeast, PTS1- and PTS2-dependent protein targeting pathways are independently mediated by Pex5p and Pex7p and combine into one pathway when each peroxin binds to Pex14p (43). In contrast, Ceanorhabditis elegans is known to have no PTS2-dependent protein targeting pathway (44).

In higher plant cells, all enzymes involved in fatty acid β-oxidation and the glyoxylate cycle except cytosolic aconitase are predominantly localized in glyoxysomes (45, 46). Among these enzymes, short chain acyl-CoA oxidase, medium to long chain acyl-CoA oxidase, a multifunctional enzyme, malate synthase, and isocitrate lyase are PTS1-containing proteins, whereas medium chain acyl-CoA oxidase, long chain acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, malate dehydrogenase, and citrate synthase are PTS2-containing proteins. The loss of Pex5p resulted in reduced amounts of all these enzymes in glyoxysomes. Therefore, plants had reduced glyoxysomal function, which was confirmed by their resistance to 2,4-DB and the requirement of sucrose for post-germinative growth (Fig. 2). In addition, the effects of CO2 and measurements of maximum quantum yield of photosystem II in adult plants indicated that loss of Pex5p also reduced leaf peroxisomal function (Fig. 3). It is known that leaf peroxisomes in photosynthetic organs contain enzymes responsible for photorespiration (12). All of these enzymes are PTS1-containing proteins, whose import into leaf peroxisomes is mediated by Pex5p. A similar pleiotropic phenotype was also observed in the Arabidopsis pet2 (PEX14 orthologue) mutant that is defective in the import of both PTS1- and PTS2-containing protein (16).

In contrast, the loss of Pex7p resulted in reduced glyoxysomal function, and no significant effect was observed in leaf peroxisomal function (Figs. 2 and 3). These results suggest that leaf peroxisomal function is predominantly maintained by Pex5p. This idea is supported by our recent report (13) showing a comprehensive survey for peroxisomal gene expression in Arabidopsis. We extensively examined the expression of all these genes by using a custom-made microarray. Statistical analyses of the expression profiles revealed that peroxisomal genes could be classified into five groups. One of the groups contains genes actively transcribed in photosynthetic organs containing leaf peroxisomes, i.e. green cotyledons and leaves. They encode glycolate oxidase, hydroxyoxypyruvate reductase, serine-glyoxylate aminotransferase, and glutamate-glyoxylate aminotransferase and malate dehydrogenase. The former four enzymes, except malate dehydrogenase, are all PTS1-containing proteins and completely cover all of the reactions that are necessary for photorespiration to occur within leaf peroxisomes. This result indicates that leaf peroxisomal function is maintained predominantly by PTS1-containing proteins whose import is mediated by Pex5p. This might be a reason for why loss of Pex7p did not affect photorespiration. The only exception was malate dehydrogenase. This enzyme is a PTS2-containing protein that is believed to supply NADH that is necessary for the reaction catalyzed by hydroxyoxypyruvate reductase. However, our present results suggest that peroxisomal malate dehydrogenase might not be necessary for photorespiration and that NADH could be supplied by an alternative pathway. Overall, the results indicated that functional differentiation of leaf peroxisomes was maintained by an appropriate use of the PTS1-containing proteins.

Our statistical analyses also allow us to propose more detailed differentiation of plant peroxisomes with unknown function(s) than has been classified previously (13). Indeed, Arabidopsis mutants that had defects in PEX2 and PEX16 suggested unidentified plant peroxisomal function involved in photomorphogenesis and development of storage organelles in seeds (17, 18). There are still a number of PEX orthologues in plants whose functions have not been analyzed (15, 47). Precise analyses of the plant PEX genes are necessary to determine functional differentiation of peroxisomes occurring during the entire life cycle.

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