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THE ROLE OF PEX11 AND PEX12 PROTEINS IN PEROXISOME BIOGENESIS IN ARABIDOPSIS THALIANA

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THE ROLE OF PEX12 AND THE PEX11 PROTEIN FAMILY ON PEROXISOME BIOGENESIS IN *ARABIDOPSIS THALIANA*

By

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ABSTRACT

THE ROLE OF PEX11 AND PEX12 PROTEINS IN PEROXISOME BIOGENESIS IN *ARABIDOPSIS THALIANA*

By

Travis Lawrence Orth

Peroxisomes are simple but highly dynamic organelles found in nearly all eukaryotic organisms and their importance is exemplified by the lethal peroxisomal disorders in human. Plant peroxisomes play unique and crucial roles in governing many essential biochemical pathways that allow for the proper development and survival of plants. Peroxisome biogenesis is a coordinated event facilitated by the peroxin proteins encoded by the *PEX* genes, which mediate processes including peroxisome formation, membrane protein insertion, matrix protein import, and peroxisome division. Despite the significance of this organelle, many aspects of peroxisome biogenesis are poorly understood, especially in plants. To understand the molecular mechanisms underlying peroxisome biogenesis in plants, which are currently highly elusive, a reverse genetic approach was taken, in which we characterized several Arabidopsis genes homologous to known yeast *PEX* genes. In this thesis research, we examined the role of the Arabidopsis PEX11 protein family in peroxisome proliferation and determined that different family members are differently regulated and may have obtained distinct roles during evolution. We also performed analysis of plants in which the expression of *PEX12* gene was silenced and found that this protein is required for peroxisome formation and matrix protein import in Arabidopsis. This study supports the notion that peroxisome biogenesis machinery is conserved as well as divergent from plants to yeast and mammals.

In loving memory of Grandma Sylvia "Bunny" Orth, who inspired my education

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CHAPTER 1

Peroxisome Biogenesis Literature Review

Peroxisomes are single membrane organelles found in nearly all eukaryotic organisms. Despite their simple structure, these small dynamic organelles mediate a wide array of essential biochemical reactions. The importance of these biochemical reactions is exemplified by the lethal phenotype seen in both plants and mammals that lack peroxisomes (Fan et al., 2005; Hu et al., 2002; Oglesbee, 2005). Additionally, the great number of mammalian genetic diseases caused by dysfunctional peroxisomes provides further evidence to the importance of this seemingly simple organelle (Dirkx et al., 2005; Faust et al., 2005; Gould and Valle, 2000).

Among the many biochemical reactions facilitated by peroxisomes, lipid metabolism, specifically the oxidation of long-chain fatty acids, is one of the most important roles of peroxisomes (Purdue and Lazarow, 2001). Unique for plants, the reactions of photorespiration, the glyoxylate cycle, nitrogen metabolism, and the synthesis of plant hormones are reactions that are all dependent on functionally competent peroxisomes (Hayashi and Nishimura, 2003). A byproduct of some of these reactions is harmful reactive oxygen species such as hydrogen peroxide. The metabolism of hydrogen peroxide is another very important role of peroxisomes, which uses the enzyme catalase to convert hydrogen peroxide into water and oxygen to prevent any cellular damage by this very reactive compound (Schrader and Fahimi, 2004). The sessile nature of plants makes it extremely important that they be able to cope with all of the different stresses and physiological conditions of their environment. Many of the reactions conducted by peroxisomes aid in the plant's ability to cope with the large array of stresses and physiological conditions that they experience. Peroxisomes are key

components to the adaptability of plants based on the versatile properties of this important organelle.

The great plasticity of peroxisome numbers within eukaryotic cells is facilitated by a group of genes that encode the peroxin (PEX) proteins. Approximately 32 *PEX* genes have been identified in yeast that are responsible for peroxisome biogenesis (Heiland and Erdmann, 2005). Approximately 20 mammalian and 15 plant proteins contain homology with the yeast PEX proteins (Charlton and Lopez-Huertas, 2002; Purdue and Lazarow, 2001) The PEX proteins are responsible for regulating all aspects of peroxisome biogenesis, including assembly of new membrane structures, peroxisome membrane protein (PMP) targeting, matrix protein import, division, and proliferation. Although numerous studies on peroxisome biogenesis have occurred in both mammals and plants, the vast majority of information on peroxisome biogenesis has been developed in the yeast systems. Therefore, the model of peroxisome biogenesis that will be summarized here is derived primarily from yeast studies. Unless noted, it can be assumed that the system being described is of yeast origin.

Peroxisome Formation

It has long been hypothesized that peroxisomes are initially derived from membranous structures budding off of the endoplasmic reticulum (ER) (Novikoff and Novikoff, 1972). These early studies relied on electron microscopy (EM) pictures to observe the close association of peroxisomes with the ER. Until recently, biochemical and cell biological techniques were not available to confirm these original observations and the theory that peroxisomes were derived from the ER was extremely tentative

(Lazarow and Fujiki, 1985). The study conducted by Hoepfner et al. (2005) using the *Saccharomyces cerevisiae* system seemed to have finally resolved the controversy associated with this issue of ER-derived peroxisome formation, at least in yeast. They implemented the use of two different forms of green fluorescent protein, cyan fluorescent protein and yellow fluorescent protein, to elucidate the action of PEX19 and PEX3 in the budding of membrane vesicles from the ER. In their study they were able to visually observe the movement of diffused PEX3 into concentrated foci in the ER before budding off in a PEX19-dependent manner to form early peroxisomal structures. These early peroxisomal structures are not fully mature competent peroxisomes and need additional PMPs as well as matrix proteins to be incorporated before they become functional peroxisomes. This study, in addition to revealing the origination of peroxisomes in yeast, has also revealed the ability for this organelle to regenerate and therefore dampened previous support for the theory that peroxisomes evolved from an endosymbionic acquisition during evolution (Latruffe and Vamecq, 2000).

Targeting Integral Membrane Proteins to the Peroxisome Membrane

Compared to matrix protein import, far less is understood about the process in which PMPs are targeted and inserted into the peroxisome membrane. What is known is that mutants with defective matrix protein import via the peroxisome targeting signal (PTS) 1 and PTS2 signals are still able to incorporate PMPs into the peroxisome membrane (Heiland and Erdmann, 2005; Santos et al., 1988). Like peroxisomal matrix proteins, PMPs are synthesized primarily on free cytosolic ribosomes and then targeted to the peroxisome (Sparkes and Baker, 2002). The hydrophobic character of integral

membrane proteins necessitates the presence of chaperones to facilitate proper folding of the proteins in the cytosol and transport of folded proteins (Schliebs and Kunau, 2004). Three PEX proteins, PEX19, PEX3, and PEX16 are believed to play a role in this process.

PEX19 is considered to be the receptor for most PMPs and *Hansenula polymorpha pex19* mutants caused a dysfunction in the development of peroxisomal structures (Otzen et al., 2004). In this same study they also found that *pex19* yeast showed a mislocalization of PMPs to other organelles, however, interestingly the overexpression of *PEX3* could rescue the mutant phenotype and allow for proper peroxisomal formation. Further studies in both human and yeast cells have verified that PEX19 is indeed a cytosolic chaperone and an import receptor for PMPs (Jones et al., 2004; Sacksteder et al., 2000). Evidence to support this role was derived from yeast two-hybrid assays showing a positive interaction between PEX19 and several PMPs including PEX10, PEX11β, PEX12, and PEX13 (Sacksteder et al., 2000). Subsequently, when a nuclear localization signal (NLS) was attached to PEX19, it caused a mislocalization of all of these PMPs to the nucleus, due to the NLS pulling PEX19 away from the peroxisomal membrane and into the nucleus (Sacksteder et al., 2000). PEX19 was not found to bind peroxisomal matrix proteins, verifying its specificity for PMP insertion into the peroxisome membrane.

PEX3 has been recently revealed to act as the docking factor for the import of PMPs into the peroxisome membrane in human cell culture experiments (Fang et al., 2004). Again, similar to *pex19* mutants, *S. cerevisiae pex3* mutants lacked detectable peroxisome membrane structures, which suggests the essential function of PEX3 in PMP

insertion and overall peroxisome biogenesis (Hettema et al., 2000). Using fluorescence recovery energy transfer to detect protein interactions, Muntau et al. (2003) discovered that PEX3 and PEX19 interact on the surface of the peroxisome membrane. This interaction is deemed essential for PMP import, since disruption of the PEX19-binding site on PEX3 eliminates the function of PEX3 (Fang et al., 2004). Moreover, it has been discovered that the PEX19/PEX3 pathway for PMP import occurs only for one class of PMP proteins, the class I PMPs. Class I PMPs are characterized by having membrane targeting signals (mPTS) that bind to PEX19 for import, whereas class II PMPs are inserted into the peroxisome membrane independently of PEX19 (Fang et al., 2004; Jones et al., 2004; Heiland and Erdmann, 2005). PEX3 is the only known class II PMP, and along with PEX16 and PEX2, have been shown to localize to the ER and to peroxisomes; however the localization of PEX3 and PEX16 to the ER is dependent on PEX19 (Kunau and Erdmann, 1998; Sacksteder et al., 2000; Titorenko and Rachubinski, 1998). Analogous studies in *Arabidopsis thaliana* have found similar results, with PEX16 localizing in both the ER and mature peroxisomes (Karnik and Trelease, 2005). However, PEX2 and PEX10 have not been found within the ER of Arabidopsis and these two proteins seem to be inserted into peroxisomes directly from the cytosol (Sparkes et al., 2005).

The role of PEX16 in PMP protein import is the least understood of the three proteins. *Yarrowia lipolytica pex16* mutants are defective in peroxisome assembly and exhibit only small electron dense structures resembling peroxisomes (Eitzen et al., 1997). However, in this same study they found that overexpressing *PEX16* in oleic acid-grown cells causes a reduced number of enlarged peroxisomes, suggesting a possible role in

peroxisome proliferation. In humans, the PEX16 protein is orientated within the peroxisome membrane with both its N and C-terminal ends exposed to the cytosol and requires the basic amino acid sequence at positions 66-81 for targeting to peroxisomes (Honsho et al., 2002). Two studies on the Arabidopsis PEX16 homologue found that *pex16* mutants were defective in peroxisome formation and contained impaired fatty acid synthesis, causing a lethal shrunken seed phenotype (Lin et al., 1999; Lin et al., 2004). This shrunken seed phenotype is the result of a build up of high levels of starches and extremely reduced amount of lipids due to the lack of functional peroxisomes, causing extreme desiccation during seed formation. Furthermore, it has also been shown using a yeast two-hybrid approach that PEX16 can interact with PEX19 (Fransen et al., 2001). The exact role of PEX16 in PMP import is still largely enigmatic, but it is believed that its major role is in the biogenesis of recognizable peroxisomes via insertion of other PMPs into the peroxisome membrane (Johnson and Olsen, 2001).

The mPTS targeting sequences are not defined by succinct consensus sequences as exhibited by PTS1 and PTS2 targeting sequences for the peroxisomal matrix. Rather, mPTSs are much larger, between approximately 50-100 aa in length, are mainly composed of basic residues, and typically are found near hydrophobic transmembrane regions (Brosius et al., 2002; Jones et al., 2004; Otzen et al., 2004). The lack of an mPTS consensus sequence makes it more difficult to determine the localization of predicted PMPs to the peroxisome membrane; therefore, individual characterization studies need to be conducted to confirm the localization of each PMP.

Peroxisomal Matrix Protein Targeting

Peroxisomes do not contain any genomic information; therefore, all the proteins that are required for performing biochemical reactions within peroxisomes need to be actively imported into this organelle. Peroxisome biogenesis can be greatly influenced by the metabolic demands of a cell, thus with increased metabolic demand there is a parallel increase in the synthesis and import of proteins required to conduct biochemical reactions within peroxisomes (Brown and Baker, 2003; Rottensteiner et al., 2003a). Therefore, successful protein import into the peroxisomal matrix has a great influence on the overall biogenesis of peroxisomes.

Proteins are drawn into the peroxisome matrix via two independent pathways. Each of these pathways utilizes a separate targeting signal: PTS1, a SKL tripeptide or its variants, found on the extreme C-terminal end of proteins, as well as a PTS2, nonapeptide comprised of the RLX_5HL sequence or its variants, found in the N-terminal region of proteins (Lazarow, 2003). Two PEX proteins, PEX5 and PEX7, act as soluble receptors for proteins containing PTS1 and PTS2 targeting signals respectively (Titorenko and Rachubinski, 2001). These two receptors are essential for binding and transporting peroxisomal matrix enzymes that are synthesized in the cytosol on free ribosomes to the peroxisome. Within the last few years there have been many important discoveries on the process in which proteins are targeted to the peroxisome and are translocated through the peroxisomal membrane and into the matrix. Much of this research has focused on the PTS1-containing proteins due to the greater abundance of PTS1-containing proteins compared to PTS2-containing proteins (Purdue and Lazarow, 2001). The PEX5 protein contains a 7 tetratricopeptide repeat motif at its C-terminus that is able to interact with the PTS1 signal, which allows for binding of PTS1-containing proteins to the PEX5 receptor

(Klein et al., 2001). Alternative splicing of the PEX5 transcript produces a long and a short variant in mammalian species (Braverman et al., 1998). The long variant, PEX5pL, has been shown to influence PTS2 protein targeting through a direct interaction with the PEX7 protein (Otera et al., 2000). Thus, mutations that disrupt synthesis of PEX5pL cause an inhibition of import of both PTS1- and PTS2-containing proteins. The plant PEX5 protein does not undergo alternative splicing and most closely resembles the long variant (Johnson and Olsen, 2001), and was found to influence the import of both PTS1- and PTS2-containing proteins (Woodward and Bartel, 2005).

Once a peroxisome-targeted protein is bound to its receptor, it is then brought to the surface of the peroxisome via an interaction with other PEX proteins. Both PEX5 and PEX7 can interact with the PEX14 protein located on the peroxisomal membrane, and mutations within PEX14 have been shown to disrupt this interaction (Albertini et al., 1997). Additionally, PEX5 has been shown to be able to interact with the SH3 domain of PEX13 to initiate the PTS1 protein import process (Gould et al., 1996). However, PEX14 is the only protein that has been shown to be directly involved with interacting with the PEX7 receptor. Further studies have shown that a third protein, PEX17, is also involved with the import of matrix proteins. *S. cerevisiae* containing a mutated PEX17 protein were not able to import either PTS1- or PTS2-containing proteins and PEX17 was shown to directly interact with PEX14 and indirectly with PEX5 through an association with PEX14 (Huhse et al., 1998). Finally, protein interaction studies have found that PEX14 is able to interact with itself, forming a homodimer within the receptor binding complex (Albertini et al., 1997).

Once bound to the PEX14 docking complex, peroxisomal matrix proteins are imported to the matrix via a second group of proteins that permits protein translocation across the peroxisomal membrane. The exact process in which this occurs remains largely enigmatic. However, several *PEX* genes have been identified and characterized using a variety of methods to show their essential role in the import of peroxisomal matrix proteins. The three proteins PEX2, PEX10, and PEX12 are all RING (really interesting new gene) finger domain-containing proteins believed to be key players in translocation steps of peroxisome protein import (Titorenko and Rachubinski, 2001). Null mutations in any of these proteins in Arabidopsis cause an embryo lethal phenotype, displaying the essential role of each of these individual proteins in plant peroxisome biogenesis and embryogenesis (Fan et al., 2005; Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003).

In addition to the RING finger protein complex, PEX8 has also been shown to be involved with protein import. Agne et al. (2003) used co-immunopurification techniques to show that PEX8 is required to organize the association between the PEX14 docking complex and the RING finger protein import complex. The complete association between these two complexes to form a functional protein import aparatus has been coined the Importomer (Kragt et al., 2005). The essential role of PEX8 in the formation of the Importomer is exemplified by the lack of protein import into peroxisomes in *S. cerevisiae* cells lacking PEX8 (Rehling et al., 2000). Steps following the formation of the Importer in protein import are still largely unknown. However, recent studies have begun to elucidate some of the steps involved with the actual translocation of peroxisome targeted proteins. One study by Collins et al. (2000) used the power of epistatic analysis

to help determine the sequence of PEX protein utilization during peroxisomal matrix protein import, downstream from the RING peroxins. In their study they showed that PEX4, PEX22, PEX1 and PEX6 act in the terminal steps of import. Additional studies have shown that PEX4 acts as an ubiquitin-conjugating enzyme and is involved with the addition of ubiquitin moieties to PEX5 (Platta et al., 2004; Kragt et al., 2005; van der Klei et al., 1998). The ubiquitination process involves the sequential action of three enzymes: an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) (Pickart, 2001). Many E3 ligases utilize their RING finger catalytic domain to facilitate the transfer of an ubiquitin moiety from the E2 conjugating enzyme to the target protein (Pickart, 2001). Therefore, the structural homology of the RING finger-containing proteins of PEX2, PEX10 and PEX12 to some E3 ligases, along with the interaction between PEX4 and PEX10, implicate that these two sets of proteins may be acting to ubiquitinate the PEX5 receptor and possibly other peroxisomal proteins in a classical ubiquitin attachment cascade (Eckert and Johnsson, 2003). However, no evidence has yet been presented showing PEX10 or the other RING finger-containing PEX proteins to act as a canonical E3 ligase. Although PEX10 has not yet been confirmed to be an E3 ligase, studies in multiple yeast species have shown that mono-ubiquitination of PEX5 causes its translocation to the peroxisomal membrane and is essential for proper protein import into the peroxisomal matrix (Platta et al., 2004; Kragt et al., 2005; van der Klei et al., 1998). Null mutations of PEX4 cause an accumulation of polyubiquitinated forms of PEX5, which previous studies have shown is a signal for degradation by the proteasome (Thrower et al., 2000). This polyubiquitination involves a PEX4-independent ubiquitination by the ubiquitin-conjugating enzyme Ubc4p, that possibly acts to remove

un-used or non-functional PEX5 receptors from the docking site (Krag et al., 2005; Kiel et al., 2005). The PEX4 protein is anchored to the peroxisomal membrane by the PEX22 protein (Koller et al., 1999). Cells lacking PEX22 exhibit a decreased interaction between PEX4 and PEX10 (Eckert and Johnsson, 2003). Overall, the mono-ubiquitination of PEX5 seems to act as a regulatory step to facilitate the turnover of this receptor to the cytosol after a PTS1-containing protein has been imported to the peroxisomal matrix.

Proteins lacking a distinguishable PTS and several proteins with their PTS1 signal removed were still able to localize to the peroxisome matrix (Sparkes and Baker, 2002). How does this targeting occur without a canonical peroxisome import signal? Sparkes and Baker (2002) hypothesized that alternative non-PTS1 interactions could be occurring with PEX5, or that some of these proteins could be 'hitch hiking' along with proteins that contain a targeting signal into the peroxisome matrix.

The extensive research devoted to the import of proteins into the peroxisomal matrix has led to multiple theories that attempted to summarize the steps of this process. Conflicting results to the subcellular location of the PEX5 receptor has provided the basis for deriving the simple shuttle and the extended shuttle models of protein import (Smith and Schnell, 2001). Recently the extended shuttle model, in which the PEX5 receptor actually travels with the PTS1-containing protein into the matrix (Szilard et al., 1995; Zhang and Lazarow, 1996), has gained increasing popularity and is currently the more accepted of the two models (Kunau, 2001). Moreover, experiments have now been initiated to elucidate the mechanistic properties to the extended shuttle model. Two recent studies using mammalian systems have found that the cycling of the PEX5

receptor from the cytosol to the matrix and back to the cytosol is ATP-dependent and is also dependent on the N-terminal 110 amino acids (Costa-Rodrigues et al., 2004; Oliveira et al., 2003). Lastly, the AAA (ATPase associated with a variety of cellular activities) peroxins PEX1 and PEX6 have been shown to play an essential role in the release of PEX5 back into the cytosol (Platta et al., 2005). The transient pore model proposed by Erdmann and Schliebs (2005) is the latest model that fully encompasses the recent literature on peroxisomal protein import. Their model includes the three consecutive steps of matrix protein import: the formation of a translocation pore, the mono-ubiquitylation of the import receptors, and the pore disassembly and subsequent receptor recycling, summarized here in Figure 1.1. With this model they also note the similarities between peroxisome and nuclear protein import (Pemberton and Paschal, 2005) in that completely folded or oligomeric proteins can be imported into peroxisomes. In contrast, only unfolded and monomeric proteins are imported into chloroplast and mitochondria (Rehling et al., 2004; Stoll and Schleiff, 2004).

ER Independent Peroxisome Proliferation in Yeast

Formation of new peroxisomes from the ER membrane is not the only type of regeneration exhibited by peroxisomes. It has long been observed that peroxisome numbers are very malleable and can change depending on the needs of a particular cell (Lazarow and Fujiki, 1985). This plasticity in peroxisome numbers has also been found to be influenced by the autonomous proliferation of peroxisomes independent of the ER (Yan et al., 2005). Of the PEX proteins identified in a variety of species, PEX11 is the only protein found to display functional similarity involving

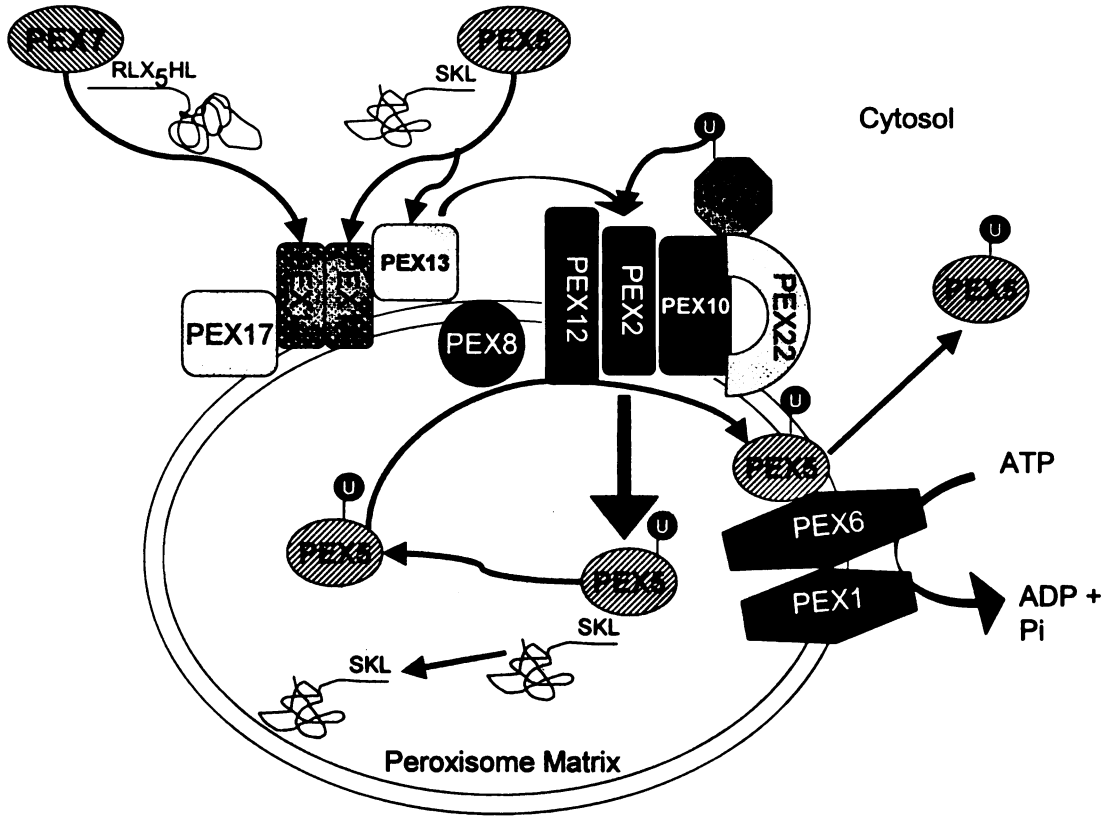

Figure 1.1. Schematic Model for Peroxisome Matrix Protein Import in Yeast. PTS1-containing proteins are first bound by PEX5 which is then able to interact with both PEX14 and PEX13. After binding to the periphery of the peroxisome membrane, PEX5 is transferred to the RING finger protein complex where it is imported along with the PTS1-containing protein. Once inside the peroxisome the PTS1-containing protein and PEX5 disassociate and PEX5 is then transferred out of the peroxisome in an ATP-dependent manner via the AAA ATPase proteins PEX6 and PEX1. Once in the cytosol, PEX5 can then be reused or degraded by the proteosome. This model is based on an interpretation of the literature discussed in this thesis.

peroxisome proliferation across several species. PEX11 has been found to induce proliferation in a range of species including humans (Abe and Fujiki, 1998; Tanaka et al. 2003;), rat (Schrader et al., 1998), mouse (Li and Gould, 2002; Li et al., 2002a; Li et al., 2002b), *Trypanosoma brucei* (Lorenz et al., 1998; Maier et al., 2001) and *S. cerevisiae* (Erdmann and Blobel, 1995; Marshall et al., 1995). Although the PEX11 protein has homologues in several species, the amino acid sequence does not display any recognizable functional motifs, except for a dilysine motif, which will be discussed later. Additionally, all of the PEX11 proteins characterized to date localize to peroxisomes.

Normally when yeast cells are transferred to a media containing a sole carbon source such as oleic acid, which requires metabolism by peroxisomes, a proliferation of peroxisomes is observed to allow for continued growth on this alternative carbon source (Veenhuis et al., 1987). Original studies showed an inhibition of growth of *S. cerevisiae pex11* mutants while on oleic acid media and that these cells contained only a few very large peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995). The presence of very large but few peroxisome in *pex11* mutant cells suggests that PEX11 plays a direct and positive role in the division and proliferation process of peroxisomes in yeast. Furthermore, the overexpression of *PEX11* caused an increase in peroxisome numbers, thus further supporting the role of PEX11 in peroxisome proliferation (Marshall et al., 1995).

Transcriptome profiling of genes induced by oleic acid has found additional proteins involved with peroxisome proliferation (Smith et al., 2002). One specific gene identified in this screen was *PEX25*. Further characterization of *PEX25* found that it played an intimate role in promoting peroxisome proliferation and that its homolog,

PEX27, also plays a role in peroxisome proliferation (Rottensteiner et al., 2003b; Tam et al., 2003). In the studies on PEX25 and PEX27 it was found that in *S. cerevisiae* *pex11pex25pex27* triple mutants the utilization of long-chain fatty acids as a carbon source was lost. This phenotype could be partially complemented with any of the three genes. Additionally, a yeast two-hybrid analysis revealed that all three of the proteins can form homodimers and PEX25 can also weakly interact with PEX27. These results suggest that the two additional *PEX25* and *PEX27* genes may be part of a larger *PEX11*-type gene family in yeast. Furthermore, PEX30, PEX31, and PEX32 are also peroxisomal integral membrane proteins containing two, four, and six transmembrane spanning regions respectively, which have been shown to regulate peroxisome size and numbers within yeast (Vizeacoumar et al., 2004). Identification of these proteins in *S. cerevisiae* was accomplished through a homology based screen with each of the proteins displaying a high degree of homology to the *Y. lipolytica* PEX23 protein. PEX23 of *Y. lipolytica* was shown to be involved in peroxisome biogenesis but its role in peroxisome proliferation is not well established (Brown et al., 2000). The study of *PEX30*, *PEX31* and *PEX32* genes showed that mutations in any one of these genes and also double and triple mutants showed no defects in growth on oleic acid media. However, a distinct phenotype was observed in the peroxisome number and morphology when observed using EM, with *pex30* exhibiting an increase numbers of peroxisomes and *pex31* and *pex32* showing both an increase in numbers along with enlargement of peroxisomes compared to WT cells (Vizeacoumar et al., 2004).

The mechanistic role that each of these proteins plays in the actual division process of peroxisomes is still largely unknown. An equally mysterious aspect of

peroxisome proliferation is how all the genes responsible for this process are regulated under conditions that require increased abundance of peroxisomes. In yeast, this regulation has been found to be primarily dependent on fatty acid content of the cells. The exposure of certain fatty acids such as oleate causes the upregulation of *PEX25* transcript via the binding of the Pip2p-Oaf1p heterodimeric transcription factor that binds to the oleate response element-like sequence within the *S. cerevisiae PEX25* promoter (Rottensteiner et al., 2003a). The Pip2p-Oaf1p transcription factor was shown to co-regulate *PEX11* gene expression along with the transcription factor Adr1p (Gurvitz et al., 2001). *S. cerevisiae adr1* mutant cells displayed a similar phenotype to that seen in *pip2* and *oaf1* mutant lines in that the number and size of peroxisomes was radically reduced (Rottensteiner et al., 1996). These results suggest that in yeast, one of the main functions of the PEX11-PEX25 class of proteins is for the initiation of peroxisome proliferation in response to a high fatty acid content carbon source. No nuclear proteins homologous to these yeast transcription factors have yet been identified in plants.

Peroxisome Proliferation in Mammals Mediated by the PEX11 Gene Family

The mammalian species, including humans, rats, and mice, contain a family of three *PEX11* genes: *PEX11α*, *PEX11β*, and *PEX11γ* (Li et al., 2002a; Schrader et al., 1998; Tanaka et al., 2003). The proliferation effect on peroxisomes by the overexpression of *PEX11* in yeast was originally believed to be simply a result of increased medium-chain fatty acid oxidation and its role in proliferation was merely secondary (van Roermund et al., 2000). However, further studies in mice examining the role of *PEX11β* discovered that the exclusive role of PEX11 proteins is in peroxisome

proliferation and division (Li and Gould, 2002). Through certain control experiments which limited the levels of medium-chain fatty acid oxidation and lipid metabolism in general, it was shown that peroxisomes could still undergo proliferation events even in the absence of metabolic activity. The presentation of this data solidified the role of PEX11 proteins as being exclusively involved in peroxisome proliferation.

The individual roles that each of the PEX11 proteins play in mammalian species vary greatly. *PEX11β* is constitutively expressed within all tissues in rats and mice, suggesting that it plays a role in the overall maintenance of peroxisome numbers within all tissues (Li et al., 2002b; Schrader et al., 1998). Overexpression of *PEX11β* in human HepG2 cells resulted in a drastic increase in peroxisome numbers (Schrader et al., 1998). Homozygous knock-out mice of *PEX11β* displayed a slight reduction in overall peroxisome number along with increased clustering and elongation of the peroxisomes that were present in these mice (Li et al., 2002b). Despite the altered morphology, these peroxisomes were metabolically functional in that they were able to import matrix proteins via the PTS1 and PTS2 pathways. Nonetheless, these knock out-mice did display neurological defects that resembled mouse models of Zellweger syndrome and only survived one day after birth.

The second mammalian *PEX11* gene, *PEX11α*, has a much different expression pattern than *PEX11β*, in that it is expressed very highly in tissues such as the liver and kidney and is barely detectable in other tissues of rats and mice (Li et al., 2002a; Schrader et al., 1998). This type of expression profile suggests that *PEX11a* plays specific roles for inducing peroxisome proliferation in certain tissues where peroxisomes are more essential, such as the kidney and liver. Overexpression of *PEX11a* in human HepG2 cells

exhibited increased peroxisome proliferation, but to a lesser extent compared to the overexpression of *PEX11β* (Schrader et al., 1998). Likewise, *PEX11a* knock-out mice also did not show drastic physiological or developmental defects or altered numbers of peroxisomes within individual cells (Li et al., 2002a). When the *PEX11a*$^{-/-}$ mice were crossed with the *PEX11β*$^{-/-}$ to produce a double knockout mouse, the phenotype observed was nearly identical to the *PEX11β*$^{-/-}$ mice.

Far less is understood about the role that the third *PEX11* gene, *PEX11γ*, plays in the process of peroxisome proliferation. Similar to the expression pattern of *PEX11α*, expression of *PEX11γ* was tissue specific, with the highest levels observed in the liver (Li et al., 2002a). Overexpression of *PEX11γ* did not show any discernable effect on peroxisome proliferation, but exhibited a slight increase in tubulation, enlargement, and clustering (Li et al., 2002a). A knock-out-mouse has yet to be generated for this gene and the expression level of *PEX11γ* did not increase as a compensatory function in the *PEX11β*$^{-/-}$ or *PEX11α*$^{-/-}$ single knock-out or double knock-out mouse.

A significant difference between *PEX11α* and the other two *PEX11* genes found in mammalian species is the responsiveness of *PEX11α* to peroxisome proliferating agents (Abe et al., 1998; Li et al., 2002a). It has long been known that certain compounds such as rodent hepatocarcinogens and fibric acid derivatives cause peroxisome proliferation and about 15 years ago the first peroxisome proliferating activated receptor (PPAR) was cloned and characterized (Hess et al., 1965; Issemann and Green, 1990; Kieć-Wilk et al., 2005). PPARs comprise a family of three proteins in humans which are each able to form heterodimers with the 9-cis retinoic acid receptor (RXR) and act as nuclear hormone receptors (Schoonjans et al., 1996). This PPAR/RXR

heterodimeric nuclear hormone receptor is activated by a vast array of peroxisome proliferating agents, translocates to the nucleus where it can bind to peroxisome proliferating response elements (PPREs) in target genes (Kliewer et al., 1992). PPREs are present in the promoters of *PEX11α* (Shimizu et al., 2004) and many other genes encoding matrix proteins associated with fatty acid metabolism (Mandard et al., 2004).

PEX11α contains one PPRE; however, unlike most PPREs, it is located downstream of the *PEX11α* open reading frame (Shimizu et al., 2004). The *PEX11α* PPRE also lies upstream from the perilipin gene and has subsequently been shown to be differentially activated by PPARα and PPARγ2 in liver and adipose tissue respectively. The absence of any PPRE elements in the regulatory region of the *PEX11β* and *PEX11γ* open reading frames explains why neither of these two genes are responsive to peroxisome proliferating agents. However, in *PEX11α*$^{-/-}$ mice peroxisome proliferation was still observed in response to each of the three PPARα activators: ciprofibrate, WY-14,643, and Di(2-ethylhexyl)phthalate (Li et al., 2002a). Additionally, a PPARα-independent factor 4-phenylbutyrate was found to be able to induce peroxisome proliferation via *PEX11α*. These results in concert orchestrate how *PEX11α* may be influencing peroxisome proliferation both independent and dependent of PPARα. So far no PPAR-homologous sequence has been identified in plants.

Dynamin-related Proteins Involved with Peroxisome Proliferation

Dynamin proteins consist of a large superfamily of proteins that act to facilitate budding of clathrin-coated vesicles during vesiculation events through the use of their GTPase capacities (Takei et al., 2005). Included in this family are dynamin-like proteins,

which are functionally similar to 'classical' dynamins, except that they lack the proline rich domains found in the 'classical' dynamin proteins (Praefcke and McMahon, 2004). One specific dynamin-like protein found to play a role in peroxisome proliferation is dynamin-like protein 1 (DLP1), which has been known to be involved in mitochondrial division (Pitts et al., 2004). Li and Gould (2003) have found that DLP1 plays an essential role in peroxisome proliferation in mammals. In their study they found that when a dominant negative form of DLP1 was expressed in human fibroblast cells there was a significant reduction of peroxisomes within these cells. Furthermore, in cells co-expressing DLP1 siRNA and PEX11-overexpressing constructs the proliferative effect of overexpressing the *PEX11* family members was negated, and only peroxisome elongation was observed. Lastly, overexpression of *PEX11β* was shown to initiate the recruitment of DLP1. A similar study by Koch et al. (2004) observed that silencing of DLP1 in COS-7 cells inhibited separation of peroxisomes and resulted in a segmented morphology. Yeast with mutant versions of Vps1p, the yeast homologue of DLP1, did not show an elongation effect but did form a few very large peroxisomes per cell (Hoepfner et al., 2001). Again, this result suggests that cells lacking certain dynamin-related proteins are not able to induce fission of peroxisomes. A plant mutant screen looking for Arabidopsis mutants displaying aberrant peroxisome morphology identified a dynamin-related protein, DRP3A (Mano et al., 2004). Arabidopsis plants containing mutations within DRP3A had an overall reduction in peroxisome numbers and peroxisomes displayed an extremely elongated morphology. Overall plant growth of *drp3a* plants was significantly reduced as compared to WT plants. The noticeable phenotype exhibited by these mutant

plants suggests that among the 16 dynamin-related proteins in Arabidopsis (Hong et al., 2003), DRP3A plays a unique role in peroxisome division and plant growth in general.

The Mechanism of Peroxisome Division

Unlike chloroplast and mitochondrial division (Osteryoung, 2001), not much is known about the actual mechanism of peroxisome division. One of the most distinguishing aspects of the PEX11 proteins is the presence of a dilysine motif (KXKXX) at the extreme C-terminal region of the mammalian *PEX11α* protein (Schrader et al., 1998). Dilysine motifs have been suggested to play a role in targeting proteins to the ER through an interaction with ADP ribosylation factor and COP coatomer proteins (Letourneur et al., 1994, Zhao et al., 1999). It has been shown that the dilysine motif of *PEX11α* is able to interact with coatomer proteins and that peroxisome proliferation via *PEX11α* induction is obstructed in ε-COP mutant Chinese hamster ovary cells (CHO) (Passreiter et al., 1998). Moreover, a similar study found that elongation and tubulation of peroxisomes occurred in CHO cells containing the ε-COP mutation (Anton et al., 2000). In contrast, the trypanosome PEX11 protein contains a non consensus KIK C-terminal motif and it is still able to bind coatomer proteins (Maier et al., 2000). When this motif was mutated, the ability to bind coatomer proteins was not diminished. Research focusing on the role of the dilysine motif concerning peroxisome proliferation is extremely tentative at this point and the role of the dilysine motif of PEX11 proteins is still yet to be determined.

The distribution and movement of peroxisomes within a cell is highly dependent on cytoskeletal elements. In mammals, microtubule elements facilitate the movement of

peroxisomes but in plants and yeast this movement is dependent on actin filaments (Mathur et al., 2002; Rapp et al., 1996). Interestingly, the movement of plant peroxisomes is driven by myosin motors and it is speculated that this movement may play a role in the fission of preexisting peroxisomes (Jedd and Chua, 2002; Mano et al., 2002). A study using human fibroblast has also found that inhibition of peroxisome motility disrupts peroxisome biogenesis (Brocard et al., 2005). Lastly, a study in *S. cerevisiae* found that the GTPase Rho1p is recruited to peroxisomes by PEX25 and is required for proper peroxisome biogenesis through the dynamic assembly and disassembly of actin on peroxisome membranes (Marelli et al., 2004).

Although studies have been initiated to examine the mechanistic aspects of peroxisome biogenesis, there is a great deal that is unknown on how this single-membrane organelle undergoes division.

Peroxisome Degradation – Pexophagy

The recycling of cellular constituents from damaged organelles to be used for the maintenance of cellular homeostasis is accomplished through a process called autophagy (Baehrecke, 2005). The act of peroxisomal degradation via an autophagic process has been termed pexophagy (Farré and Subramani, 2004; Hutchins et al., 1999). This process is essential for maintaining the proper number of peroxisomes per cell depending on the metabolic demands of each cell. Pexophagy is greatly initiated when yeast cells are transferred from media containing an exclusively fatty acid carbon source to a media rich in glucose (Gunkel et al., 1999). This result is a great example of how peroxisome degradation is initiated once the function of peroxisomes is no longer essential for

Table 1.1. Functions of Yeast Peroxins and Their Arabidopsis Homologs.

Peroxin	Characteristics and Function	Arabidopsis Homologue	Citation
PEX1	AAA (ATPase associated with a variety of cellular activities), aids in the release of the PEX5 receptor to the cytosol	YES	Lopez-Huertas et al., 2000
PEX2	RING finger-containing protein, member of the import complex	YES	Hu et al., 2002
PEX3	Acts as a docking factor in the import of PMPs into the peroxisome membrane	YES	Hunt and Trelease, 2004
PEX4	Ubiquitin-conjugating enzyme, adds ubiquitin moieties to PEX5	YES	Zolman et al., 2005
PEX5	Soluble receptor for PTS1 containing proteins	YES	Johnson and Olsen, 2001
PEX6	AAA (ATPase associated with a variety of cellular activities), aids in the release of the PEX5 receptor to the cytosol	YES	Kaplan et al., 2001
PEX7	Soluble receptor for PTS2 containing proteins	YES	Woodward and Bartel, 2005
PEX8	Facilitates the association of the PEX14 docking complex and the RING finger protein import complex	NO	Rehling et al., 2000
PEX10	RING finger containing protein, member of the import complex	YES	Sparkes et al., 2003
PEX11	Peroxisome proliferation	YES	Charlton and Lopez-Huertas, 2002
PEX12	RING finger-containing protein, member of the import complex	YES	Fan et al., 2005
PEX13	Assists in PTS1-containing protein import	YES	Charlton and Lopez-Huertas, 2002
PEX14	Receptor for PEX5 and PEX7 on the peroxisomal membrane	YES	Hayashi et al., 2000
PEX15	Recruits PEX6 to the peroxisome membrane	NO	Birschmann et al., 2003

PEX16	Peroxisome membrane assembly, also believed to act as a chaperone for PMPs	YES	Lin et al., 2004
PEX17	Member of the PEX14 import receptor binding complex	YES	Charlton and Lopez-Huertas, 2002
PEX19	Targets class I PMPs to the peroxisome membrane, also acts in the vesiculation of ER membrane to form new peroxisomes	YES	Charlton and Lopez-Huertas, 2002
PEX22	Anchors PEX4 to the peroxisome membrane	YES	Zolman et al., 2005
PEX25	Peroxisome proliferation	NO	Rottensteiner et al., 2003b
PEX27	Peroxisome proliferation	NO	Rottensteiner et al., 2003b
PEX28	Regulation of peroxisome proliferation	NO	Vizeacoumar et al., 2003
PEX29	Regulation of peroxisome proliferation	NO	Vizeacoumar et al., 2003
PEX30	Regulation of peroxisome proliferation	NO	Vizeacoumar et al., 2004
PEX31	Regulation of peroxisome proliferation	NO	Vizeacoumar et al., 2004
PEX32	Regulation of peroxisome proliferation	NO	Vizeacoumar et al., 2004

Table 1.1. Cont.

growth. Pexophagy is also induced by other environmental stimuli such as cold stress, which exemplifies the plasticity of peroxisome numbers in response to environmental conditions (Komduur et al., 2004).

Significance for Peroxisome Biogenesis Research in Plants

Peroxisome biogenesis within all eukaryotic organisms is a highly regulated process. This regulation ensures that appropriate levels of peroxisome biogenesis and also proper peroxisome functioning occur in the wake of environmental stimuli and stresses. The innate plasticity involved with this regulation provides an immense level of adaptability of cells to cope with altering metabolic demands that require proper numbers of functioning peroxisomes. Although a great deal is known about many of the aspects of peroxisome biogenesis, far more is yet to be fully understood on the overall regulation of this seemingly simple organelle. The enigmatic aspect of this regulation is even more unsettled in plants. Unlike yeast and mammals, in which transcription factors have been identified to induce peroxisome biogenesis and matrix protein expression, no such upstream regulators have been identified in plants.

One could conjure that plants may not contain a similar type of overall regulation as observed in yeast and mammals for peroxisome biogenesis. However, the many complex biochemical reactions carried out exclusively by plant peroxisomes and also the responsiveness of plant peroxisomes to environmental stimuli such as light (Ferreira et al., 1989) and senescence (Lopez-Huertas et al., 2000; Pastori and del Río, 1997), makes it highly likely that a complex regulatory network of peroxisome biogenesis also occurs in plants. Furthermore, at least three distinct types of peroxisomes are displayed in plants,

leaf peroxisomes, glyoxysomes in germinating cotyledons, and nodule-specific peroxisomes, each with a unique matrix constituency that allows them to perform specific biochemical reactions (Reumann, 2000). The specialization of peroxisomes in plants allows for tissue specific allocation of peroxisomal functions, such as leaf peroxisomes which mediate photorespiratory reactions and glyoxysomes which allow for lipid metabolism during germination. Peroxisomes are present in virtually all plant tissues: their matrix composition and function in some tissues are still yet undetermined. The peroxisomal specialization observed in plants may actually suggest that plants contain multiple layers of regulation that are much more complex than those observed in yeast and mammals, which would allow for the coordination of tissue specific peroxisome biogenesis observed in plants.

Compared with the diverse biochemical pathways understood in plant peroxisomes, far less is known about how peroxisome biogenesis is regulated in plants. To begin deciphering the complex levels of regulation believed to be involved in plant peroxisome biogenesis, a thorough understanding of the function that each peroxisome biogenesis gene plays in the overall regulation of this organelle and in plant development is needed. It seems that the peroxisome biogenesis machinery is both conserved and divergent between plant and other kingdoms. The Arabidopsis genome is predicted to encode about 15 proteins homologous to some of the yeast peroxins (Mullen et al., 2001; Charlton and Lopez-Huertas, 2002); only about eight of these 15 genes have been partially characterized (Baker and Sparkes, 2005; Fan et al., 2005; Lin et al., 1999, 2004; Hayashi et al., 2000, 2005; Zolman et al., 2000; Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Zolman and Bartel, 2004;Woodward and Bartel, 2005). (Table 1.1).

This thesis research focuses on two types of such *PEX* genes, the *PEX11* gene family and the single-copy *PEX12* gene believed to be involved in peroxisome proliferation and matrix protein import respectively. A more thorough understanding of the genes responsible for peroxisome biogenesis in Arabidopsis will hopefully reveal aspects of the unique regulation of peroxisome biogenesis in plants, which is still largely unknown.

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CHAPTER 2

The Role of the *Arabidopsis thaliana* PEX11 Protein Family in Peroxisome Elongation and Proliferation

Confocal microscopy images were obtained by Jilian Fan (Figure 2.4)

Sucrose assay data was obtained with the assistance of Chie Awai (Figure 2.5)

Abstract

Peroxisomes are highly dynamic in that their numbers can change in response to a variety of developmental and environmental stimuli in yeast, mammals, and plants. However, the molecular basis for peroxisome proliferation is still largely enigmatic. PEX11 proteins from yeast and mammals have been shown to be involved in peroxisome proliferation with a yet-to-be determined mechanism. The PEX11 proteins are also the only known PEX protein to exclusively promote peroxisome proliferation across a range of species. Our study focuses on the characterization of the five putative PEX11 homologs in Arabidopsis as a starting point to understand how plants regulate peroxisome proliferation. All five PEX11 proteins when fused to the cyan fluorescent protein (CFP) were localized to the peroxisome. In addition, plants overexpressing each *PEX11* gene displayed distinct peroxisome phenotypes such as peroxisomal elongation, clustering, and increased overall peroxisome numbers. Furthermore, some members of the *PEX11* gene family showed a tissue-specific up-regulation by certain environmental stresses. Finally, phylogenetic analysis of PEX11 proteins from different species suggests that the *PEX11* gene family was amplified in plants and vertebrates after the separation of these evolutionary lineages. In summary, our data suggest that the Arabidopsis PEX11 protein family members are differentially regulated and may have acquired distinct roles in mediating various steps of peroxisome proliferation.

The dynamic and essential nature of peroxisomes within nearly all eukaryotic organisms are important characteristics for this seemingly simple, single-membrane-bound organelle. The essential nature of peroxisomes in plants is displayed by the lethal phenotype in mutant plants lacking peroxisomes and the drastic phenotype in plants with impaired peroxisomal functions (Fan et al., 2005; Hu et al., 2002; Lin et al., 1999, 2004; Schumann et al., 2003; Sparkes et al., 2003). The importance of peroxisomes in plants is further illustrated by the many essential biochemical reactions mediated by this organelle such as photorespiration, β-oxidation, the glyoxylate cycle, nitrogen metabolism, synthesis of plant hormones, and metabolism of hydrogen peroxide (Hayashi and Nishimura, 2003; Olsen and Harada, 1995). The PEX proteins are responsible for nearly all aspects of peroxisome biogenesis. PEX11 from yeast and its homologues in humans, rodents, and trapanosomes is involved exclusively in peroxisome proliferation (Subramani et al., 2000).

Studies on the PEX11 protein from yeast have shown that this protein is intimately involved with peroxisome proliferation (Erdmann and Blobel, 1995; Marshall et al., 1995; Marshall et al., 1996). It was found that (i) overexpressing *PEX11* in *Saccharomyces cerevisiae* caused a proliferative effect on peroxisome numbers, and (ii) in cells lacking PEX11 the number of peroxisomes was reduced. Additionally, these studies showed that PEX11 localizes to peroxisomes, contains two membrane spanning regions with the N- and C-termini facing the cytosol, forms homodimers for proper functioning, and is highly induced by growth on oleic acid. A recent study using *pex5* mutant human cell lines that are deficient in protein import showed that peroxisome proliferation still occurs in these metabolically inactive cells when *PEX11* is

overexpressed, therefore verifying that the primary role of PEX11 is facilitating peroxisome proliferation (Li and Gould, 2002).

Transcriptome profiling experiments in which *S. cerevisiae* were shifted from a glucose rich media to an oleate medium have identified additional proteins in yeast that seem to be intimately involved with peroxisome proliferation (Smith et al., 2002). PEX25 and PEX27 are two of these additional proteins that play somewhat parallel roles in promoting peroxisome biogenesis (Rottensteiner et al., 2003; Tam et al., 2003). PEX25- and PEX27-overexpressing yeast contained increased numbers of peroxisomes, whereas yeast cells lacking either of these two proteins displayed fewer and enlarged peroxisomes, with PEX27 exhibiting a slightly minor role in the regulation of peroxisome proliferation, compared to PEX11 and PEX25. Using the yeast two-hybrid approach, it was shown that all three proteins were able to form homodimers and that there was an interaction between PEX25 and PEX27, indicating that these proteins may form complexes that regulate peroxisome proliferation (Tam et al., 2003). More recent studies have identified *PEX28* and *PEX29* as possible negative regulators of peroxisome proliferation (Tam and Rachubinski, 2002; Vizeacoumar et al., 2003). Yeast lacking either or both of these genes contained increased numbers of peroxisomes which appear significantly smaller and clustered. The PEX28 and PEX29 proteins both localize to the peroxisome and are predicted to be integral membrane proteins with two and four transmembrane spanning regions respectively. Lastly, PEX30, PEX31, and PEX32 are also peroxisomal integral membrane proteins containing two, four, and six transmembrane spanning regions, respectively, which have been shown to regulate peroxisome size and numbers in yeast (Vizeacoumar et al., 2004). Deletion of the *PEX30*

gene results in increased peroxisome numbers, whereas deletion of the *PEX31*or *PEX32* gene results in an increased size of the peroxisomes. The plethora of genes found to regulate peroxisomes in yeast and their divergent but overlapping functions suggests that these genes may be acting in concert to regulate the proliferation, and as a result, numbers of peroxisomes, in yeast. However, the overall regulation of this process to increase or decrease peroxisome numbers in response to environmental cues has not been completely elucidated.

Like the yeast systems, mammals also contain a family of genes known to regulate peroxisome proliferation; these genes consist of the *PEX11* gene family, including *PEX11α, PEX11β,* and *PEX11γ.* Within mammals *PEX11α* and *PEX11β* seem to play independent roles in peroxisome proliferation. *PEX11α* is inducible and no discernable phenotype is observed in cells lacking this gene (Li et al., 2002a). However, *pex11β* is lethal and this gene is normally found ubiquitously expressed throughout mammalian tissues (Li eat al., 2002b). Far less is known about the role that *PEX11γ* plays in peroxisome proliferation (Li et al., 2002a; Tanaka et al., 2003). It is currently unknown how these three proteins act to regulate peroxisome numbers in mammals, but studies have shown that additional proteins such as the dynamin-like protein, DLP1, may be involved with this process (Li and Gould, 2003).

Early studies using electron microscopy techniques have shown that different environmental stimuli and the developmental state of a plant can influence the number of peroxisomes present in plant cells (Ferreira et al., 1989; Lopez-Huertas et al., 2000; Pastori and del Río, 1997). The alteration of peroxisome numbers may allow for plants

to adapt to varying environmental and developmental conditions that they experience throughout their lifetime.

Even though peroxisomes are essential for proper plant development and mediate many essential plant biochemical reactions, far less is understood on how the number of peroxisomes is regulated in plants as compared to yeast and mammals. In this study, a family of five genes in *Arabidopsis thaliana* that are homologous to the yeast and mammalian *PEX11* genes were identified and characterized. Fluorescent microscopic techniques were used to confirm the localization of each of the Arabidopsis PEX11 proteins to peroxisomes. Further microscopic, biochemical, and tissue specific expression analyses implicate these PEX11 proteins in plant peroxisome proliferation.

Material and Methods

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants used in this study are of the Columbia (Col-0) background. Seeds were germinated on 1X Muashige and Skoog medium (Gibco) after a 2-d stratification period, with or without 1% sucrose, and appropriate antibiotics when necessary. Plants used for most experiments were grown with 16/8-h light/dark photoperiod under 70 to 100 μmol m^{-2} s^{-1} light conditions at 22°C.

Sequence Alignment and Phylogenetic Analysis

The amino acid sequence alignment and the phylogenetic tree were constructed using the amino acid sequence of the PEX11 proteins from various organisms obtained from the NCBI website

(http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=Protein). The sequences were then aligned by the Clustal W method using the Megalign program from the Lasergene 6 software package (DNASTAR) and subsequently grouped into a phylogenetic tree also using this software. The neighbor-joining tree and bootstrap analysis was conducted using PAUP* 4.0 (Phylogenetic Analysis using Parsimony) (Sinauer Associates) utilizing the distance analysis function with 1000 replicates. A 50% accuracy value was used as the cut-off for branch reliability. The percent homology of the Arabidopsis PEX11 proteins compared to other species was determined using the publicly available online AliBee – Multiple Alignment software (http://www.genebee.msu.su/services/malign_reduced.html; Nikolaev et al., 1997).

Generating 35S:CFP-PEX11 and 35S:PEX11 plants

To clone 35S-CFP:PEX11, the coding region of *AtPEX11*a (At1g47750), *AtPEX11*b (At3g47430), or *AtPEX11*d (At2g45740) was amplified by RT-PCR. First-strand cDNA was made from mRNA of wild-type Columbia (Col) seedling, using primers At1g47750Fw (5'-CGCGGATCCATGGCTACGAAAGCTCCAGA-3'), At1g47750Rv (5'-CGGGGTACCTCAACAAGAGATCCAGTTCT-3'), At3g47430Fw (5'-CGCGGATCCATGTCTTTGGACACTGTGGA-3'), At3g47430Rv (5'-CGGGGTACCTCACGATGGCCAGTTCCTAT-3'), At2g45740Fw (5'-CGCGGATCCATGGGGACGACGTTAGATGT-3'), At2g45740Rv (5'-CGGGGTACCTCAGGGTGTTTTGATCTTGG-3'). The coding region of *AtPEX11c* (At1g01820) or *AtPEX11e* (At3g61070) was amplified from the cDNA clones 118F11 and 125J9, respectively, which were obtained from the Arabidopsis Biological Resource

Center (ARBC) DNA stock center. Primers used were At1g01820Fw (5'-AAACCCGGGAAATGAGTACCCTTGAGACCAC-3'), At1g01820Rv (5'-CGAGCTCTCAGACCATCTTGGACTTGG-3'), At3g61070Fw (5'-CGCGGATCCATGACTACACTAGATTTGAC-3'), and At3g61070Rv (5'-CGGGGTACCTCAAGGTGTCTTCAACTTGG-3'). The resulting RT-PCR fragments were cloned into the *Bam*HI and *Kpn*I sites or *Sma*I and *Sac*I sites at the carboxy terminus of CFP in a modified pCAMBIA1300 vector (CAMBIA) containing the 35S promoter.

To clone 35S:PEX11, the coding region of *AtPEX11*a, *AtPEX11*b, *AtPEX11*c, *AtPEX11*d, or *AtPEX11*e was amplified from the 35S-CFP:PEX11 vectors, using the primers At1g47750Fw2 (5'-ACGCGTCGACATGGCTACGAAAGCTCCAGA-3'), At1g47750Rv, At3g47430Fw2 (5'-GGGGTACCATGTCTTTGGACACTGTGGA-3'), At3g47430Rv2 (5'-CGGAGCTCTCACGATGGCCAGTTCCTAT-3'), At1g01820Fw2 (5'-GGGGTACCATGAGTACCCTTGAGACCAC-3'), At1g01820Rv, At2g45740Fw2 (5'-GGGGTACCATGGGGACGACGTTAGATGT-3'), At2g45740Rv2 (5'-CGGAGCTCTCAGGGTGTTTTGATCTTGG-3'), At3g61070Fw2 (5'-GGGGTACCATGACTACACTAGATTTGAC-3'), At3g61070Rv2 (5'-CGGAGCTCTCAAGGTGTCTTCAACTTGG-3'). The resulting PCR fragment was cloned into the *Kpn*I and *Sac*I sites or *Sal*I and *Kpn*I sites in the pCAMBIA vector containing a 35S promoter.

All PCR amplifications were carried out using the Pfu DNA polymerase (Stratagene) and protocols suggested by the manufacturer. Agrobacterium-mediated transformation of Arabidopsis plants was performed using the floral dip method (Clough

and Bent, 1998). Transgenic plants were selected on Murashige and Skoog plates containing 50 ng/μL kanamycin and 25 ng/μL hygromycin.

Epi-fluorescence and Confocal Microscopy

A Zeiss Axiophot and a Zeiss Axio Imager.M1 microscope (Carl Zeiss) were used to visualize fluorescent proteins. For in vivo detection of YFP and CFP, leaf tissue was mounted in water and viewed using Axiophot with the YFP filter (excitation 500 ± 12.5 nm, emission 540 ± 20 nm) or CFP filter (excitation 440 ± 10 nm, emission 480 ± 15 nm), and Axio Imager.M1 with the YFP filter (excitation 500 ± 12 nm, emission 542 ± 13.5 nm) or CFP filter (excitation 438 ± 12 nm, emission 483 ± 16 nm), respectively.

A Zeiss Pascal Confocal microscope (Carl Zeiss) was used to obtain confocal images of YFP proteins. Each image is an overlay of 10 cross-sections that were obtained 0.5 μm apart.

Images in this thesis are presented in color.

Sucrose Assay

Hypocotyl length of 5-d etiolated seedlings germinated on 1X Muashige and Skoog medium (Gibco) in the presence and absence of 1% supplemented sucrose was measured with a standard ruler. At least 34 plants were measured from each genotype and the experiment was repeated twice. Standard deviations for the data were calculated using the Excel program (Microsoft). Statistical significance was calculated using the Student's T-test to determine differences between hypocotyl lengths of the YFP-PTS1 control plants and the overexpressing lines on the un-supplemented media.

Yeast Two-Hybrid Analysis

The coding regions of AtPEX11a, AtPEX11b, AtPEX11c, AtPEX11d, and AtPEX11e were amplified from 35S-CFP:PEX11 vectors using the primers At1g47750Fw3 (5'-GGAATTCATGGCTACGAAAGCTCCAGA-3'), At1g47750Rv3 (5'-CCGCTCGAGTCAACAAGAGATCCAGTTCT-3'), At3g47430Fw3 (5'-GGAATTCATGTCTTTGGACACTGTGGA-3'), At3g47430Rv3 (5'-CCGCTCGAGTCACGATGGCCAGTTCCTAT-3'), At1g01820Fw3 (5'-GGAATTCATGAGTACCCTTGAGACCAC-3'), At1g01820Rv3 (5'-CCGCTCGAGTCAGACCATCTTGGACTTGG-3'), At2g45740Fw3 (5'-GGAATTCATGGGGACGACGTTAGATGT-3'), At2g45740Rv3 (5'-CCGCTCGAGTCAGGGTGTTTTGATCTTGG-3'), At3g61070Fw3 (5'-GGAATTCATGACTACACTAGATTTGAC-3'), At3g61070Rv3 (5'-CCGCTCGAGTCAAGGTGTCTTCAACTTGG-3'). The resulting fragments were cut with *Eco*RI and *Xho*I and cloned into the pGilda and pB42AD yeast expression vectors.

Yeast cells were transformed according to the methods described by Gietz and Woods (2002). Test for interaction was conducted on plates lacking appropriate amino acids according to the manufacturer's instructions. Protein expression verification was conducted using an anti-HA primary antibody (eBioscience) using a 1:400 dilution. The horseradish peroxidase secondary antibody detection system (PERBIO) was used to detect protein expression.

Plant Treatments

Cold treatments were conducted by placing 10-d seedlings in a 4° C growth chamber for 5 hours. For the hydrogen peroxide treatment 10mM H_2O_2 solution was applied to petri dishes containing ~200 leaves from 20-d Arabidopsis and soaked for 3 hours. For the NaCl treatment, 10-d seedlings were transferred to a 300 mM NaCl-saturated filter paper laying on 1X Muashige and Skoog media and also to a 250 mM NaCl supplemented 1X Muashige and Skoog media plate for 4 hours. For induced senescent treatments leaves were cut off from 20-d plants and placed in a petri dish of water and incubated at 28° C in the dark for 2 and 4 days. Natural senescence was examined by removing rosette leaves from plants grown under the conditions listed above at the specified time intervals. For the high light treatment 10-d seedlings were exposed to 4 hours of 1900 μmol m^{-2} s^{-1} light. To examine the light responsiveness, 6-d etiolated seedlings were exposed to 2 hours of 70 μmol m^{-2} s^{-1} or 1900 μmol m^{-2} s^{-1} light. Low CO_2 treatments were conducted by exposing 10-d seedlings to air containing 60 ppm CO_2 for 2 and 4 hours.

RT-PCR Analysis PEX11 Transcripts in Selected Tissues and During Stress Treatments

Total RNA was extracted with TRIzol reagent (Invitrogen) and subjected to reverse transcription (RT) reaction (Gibco). The *PEX11*-specific primers At1g47750F (5'-GCTCGTCTTACTCATAATCGC-3') and At1g47750R (5'-CATTAGGAGCCGATAACACTCC-3') were used to amplify a 391-bp product from *AtPEX11*a cDNA; At3g47430F (5'-CAGTGATCCGTTTCTTGGCG-3') and At3g47430R (5'-GGCCAGTTCCTATACCAACC-3') to amplify a 432-bp product from

*AtPEX11*b cDNA; At1g01820F (5'-TGCTCTCATTAGCCCTGTTCCC-3') and At1g01820R (5'-GGACTTGGGATGTGACGGCAAT-3') to amplify a 486-bp product from *AtPEX11*c cDNA; At2g45740F (5'-TGTCTGGCTTGGGAGATCAGGA-3') and At2g45740R (5'-TGTCTGGCTTGGGAGATCAGGA-3') to amplify a 272-bp product from *AtPEX11*d cDNA; At3g61070F (5'-GTCCTTACTCGGGAAGTCGAAG-3') and At3g61070R (5'-GATAAGTGAGGTGGTAAACC-3') to amplify a 395-bp product from *AtPEX11*e cDNA; and UBQ10-1 (5'-TCAATTCTCTCTACCGTGATCAAGATGCA-3') and UBQ10-2 (5'-GGTGTCAGAACTCTCCACCTCAAGAGTA-3') from the *UBIQUITIN*10 gene (At4g05320) to amplify a cDNA product of approximately 320-bp. PCR conditions were as follows: 94°C for 3 min, followed by 27 cycles at 94°C for 45 s, 57°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 7 min.

Results

PEX11 Amino Acid Sequence Analysis

The *Arabidopsis thaliana PEX11* gene family is comprised of five genes annotated as *PEX11*a (At1g47750), *PEX11*b (At3g47430), *PEX11*c (At1g01820), *PEX11*d (At2g45740), and *PEX11*e (At3g61070). These five genes encode proteins that share an approximately 25-30% degree of identity to the mammalian and yeast PEX11 proteins (http://www.genebee.msu.su/services/malign_reduced.html) (Figure 2.1). The only distinctive domain present in the Arabidopsis family of proteins is a dilysine motif found at the extreme C-terminal end of PEX11c, PEX11d, and PEX11e, which is also found in the mammalian PEX11α protein. The importance of this domain in proper PEX11 functioning is still not defined (Maier et al., 2000; Passreiter et al., 1998). No

other recognizable domains are present within any of the PEX11 protein sequences. Phylogenetic analysis separated the Arabidopsis PEX11 family into two distinct groups, one containing PEX11a and PEX11b, and the other containing PEX11c, PEX11d, and PEX11e (Figure 2.2). The clustering pattern observed within the phylogenetic tree of the PEX11 proteins reveals several important pieces of information about the evolution of this class of proteins. For instance, the presence of only one PEX11 protein in all fungal species examined is unique to this kingdom. However, in the other species examined, such as human, rice, and Arabidopsis, a small PEX11 protein family was present. The AtPEX11 proteins cluster independently of the human and yeast PEX11 proteins, indicating that the Arabidopsis PEX11 proteins were amplified after the separation of plant from other lineages and thus represent an evolutionarily distinct group of peroxisome division proteins. Interestingly, different members of PEX11 proteins in plant and human seem to have further amplified after the divergence of different species within the same kingdom. For example, rice PEX11-1 clusters with Arabidopsis PEX11c-e whereas OsPEX11-2 clusters with PEX11a-b, suggesting that these two groups of PEX11 proteins may perform distinct roles. Similarly, the three Human PEX11s are also separated into three independent groups with PEX11α more closely related with those from other vertebrate species. The subfamilies of AtPEX11 proteins observed within the phylogenetic tree indicate that each group of proteins may be playing discrete roles during peroxisome proliferation.

Subcellular Localization of Arabidopsis PEX11 Proteins

Figure 2.1. Amino Acid Sequence Alignment of PEX11 Proteins from Arabidopsis and Other Species.

GenBank accession numbers are as follows: *Saccharomyces cerevisiae* ScPEX11p (NP_014494); *Homo sapiens* PEX11α (NP_003838), PEX11β (O96011), PEX11γ (BAD01558); Arabidopsis AtPEX11a (NP_564514), AtPEX11b (NP_190327), AtPEX11c (NP_563636), AtPEX11d (NP_850441), and AtPEX11e (NP_191666). Sequences were aligned by the Clustal W method using Megalign from DNASTAR. Boxed regions indicate dilysine motifs.

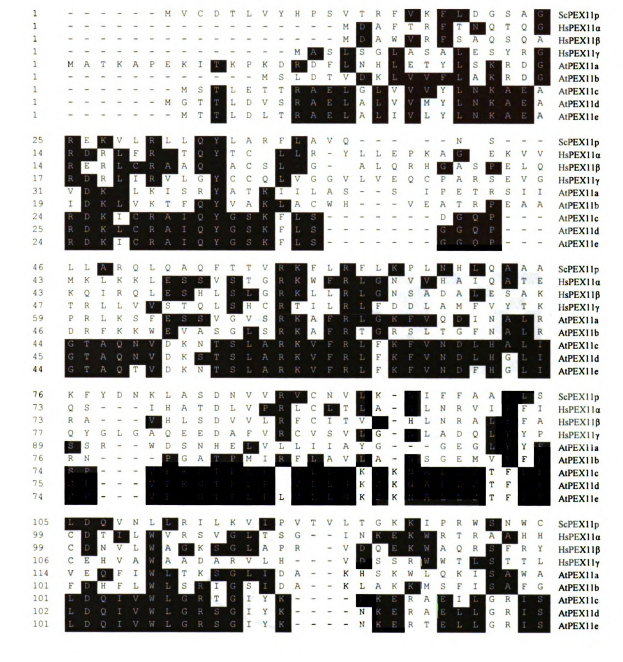
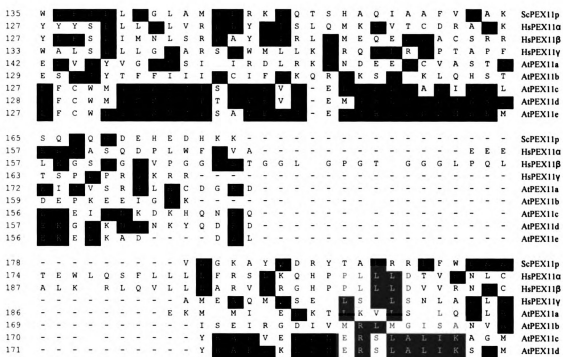


Figure 2.1. Amino Acid Sequence Alignment of PEX11 Proteins from Arabidopsis and Other Species.



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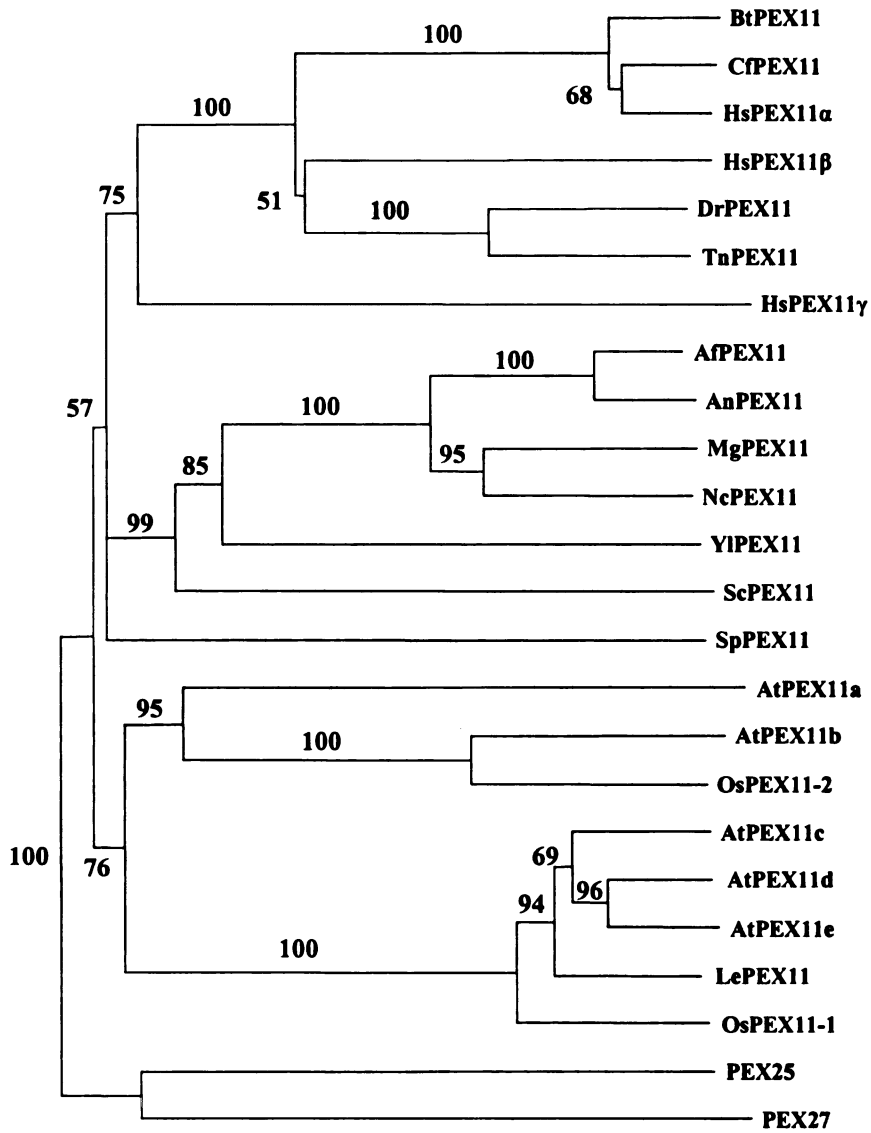


Figure 2.2. Neighbor-joining Tree, Bootstrap Analysis, of PEX11 Proteins from Arabidopsis and Other Species.

The lack of targeting sequences and divergence of the *AtPEX11* genes from their mammalian and yeast homologues and from each other prompted us to first confirm the localization of the putative Arabidopsis PEX11 proteins. To determine the subcellular localization of each PEX11 protein from Arabidopsis, we employed fluorescence microscopic analysis to Arabidopsis plants co-expressing yellow fluorescent protein (YFP)-PTS1 and cyan fluorescent protein (CFP)-PEX11 fusion proteins. Peroxisome targeting signal 1 (PTS1), the Ser-Lys-Leu (SKL) tripeptide attached to the C-terminal end of YFP, is a strong peroxisome protein import signal in both plants and other organisms (Johnson and Olsen, 2001). CFP signals were detected in most cell types in transgenic plants (data not shown). Photographs were taken from trichomes due to the more easily distinguishable CFP signal in the single-cell layer of trichomes and the ability to separate these cells away from the background fluorescence emanating from other leaf cells. For all five genes the CFP signal was in a punctate fluorescence pattern that directly overlapped with the YFP signal from YFP-SKL-enriched peroxisomes, confirming that each of the five PEX11 proteins is targeted to peroxisomes (Figure 2.3).

Peroxisome Morphology in PEX11-Overexpressing Plants

The peroxisome morphology of plants expressing each of the five *PEX11* genes under the control of the constitutively active 35S promoter was analyzed by visualization of the YFP-PTS1 fluorescence observed in 4-week-old rosette leaves. When compared to the punctate pattern of fluorescence seen in the control YFP-PTS1 plant (Figure 2.4) each of the *PEX11*-overexpressing plants displayed a uniquely altered fluorescence pattern within at least three independent lines. 35S:CFP-PEX11a and

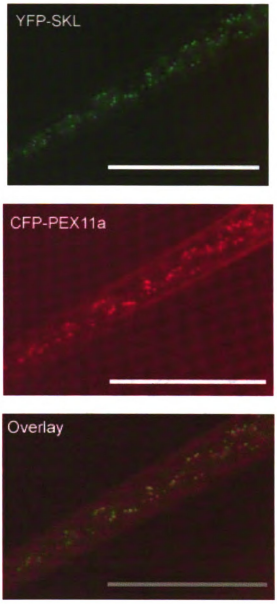
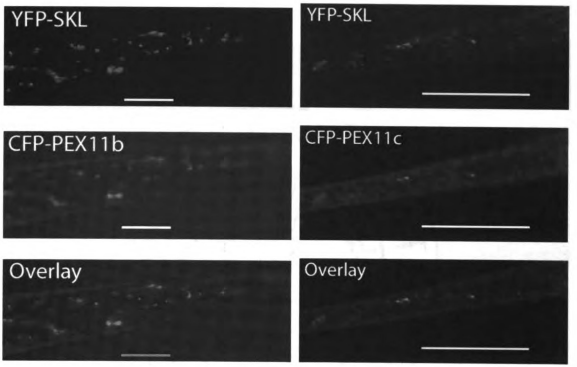


Figure 2.3. Subcellular Localization of PEX11 Proteins in Rosette Leaf Trichomes. CFP-PEX11 constructs were transfected into YFP-SKL-expressing plants to observe colocalization of the CFP-PEX11 proteins with the peroxisomal YFP signal. CFP-PEX11a, CFP-PEX11b, CFP-PEX11c, CFP-PEX11d, and CFP-PEX11e all colocalize with the YFP-SKL marker. Bars = 20μm.



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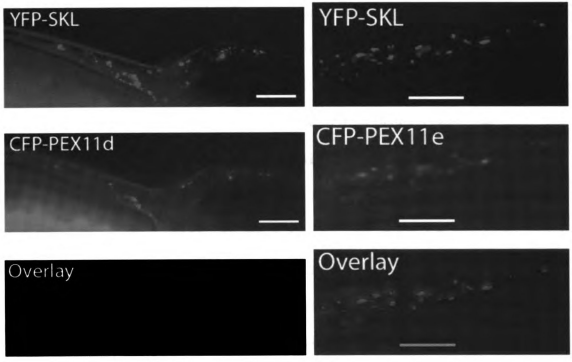


Figure 2.3. Cont.

35S:CFP-PEX11b plants both had significantly elongated peroxisomes as compared to control plants, with 35S:CFP-PEX11b often exhibiting a higher degree of peroxisome elongation (Figure 2.4). Elongated peroxisomes were also observed with 35S:CFP-PEX11c plants; however, a considerable amount of peroxisome clustering was also observed, which was not exhibited in the PEX11a- or PEX11b-overexpressing plants (Figure 2.4). Lastly, the 35S:CFP-PEX11d and 35S:CFP-PEX11e plants did not display elongated peroxisomes, but a significant amount of clustering was seen with the 35S:CFP-PEX11d plants (Figure 2.4). The amount of clustering in the 35S:CFP-PEX11e plants was reduced compared with that of the PEX11d-overexpressing plants, and the peroxisomes also seemed to be slightly enlarged in these plants (Figure 2.4). Overexpression of PEX11c, PEX11d and possibly PEX11e also seemed to produce a noticeable increase in peroxisome numbers; however, this increase was hard to definitively determine due to the extensive clustering observed. The phenotype observed with overexpressing each of the *PEX11* genes is not merely due to overexpressing a peroxisome membrane protein (PMP), given that plants overexpressing other PMPs such as PEX2 (Hu et al., 2002) and PEX12 (Fan et al., 2005) did not alter peroxisome morphology. Additionally, the overexpression of PEX11 proteins not containing a CFP tag produced consistent peroxisome morphology phenotypes that support those observed in the CFP fusion-overexpressing transgenic plants (data not shown).

Sucrose-Dependence Assay for Functional Peroxisomes

Given that peroxisome β-oxidation and the glyoxylate cycle are crucial steps in lipid mobilization, peroxisome mutants tend to develop poorly on media without

Figure 2.4. Peroxisome Phenotype Conferred by Overexpressing *PEX11* Genes in Arabidopsis.

Altered peroxisome phenotype in 35S:CFP-PEX11a, 35S:CFP-PEX11b, 35S:CFP-PEX11c, 35S:CFP-PEX11d, and 35S:CFP-PEX11e plants as compared to the control YFP-SKL plants. Epi-fluorescence pictures are shown on the left and confocal pictures are shown on the right. Bar = 20 μm for Epi-fluorescence and 5 μm for confocal pictures. An extra picture of elongated peroxisomes in a trichome cell is shown (middle photograph) for 35S:CFP-PEX11b.

Figure 2.2. Neighbor-joining Tree, Bootstrap Analysis, of PEX11 Proteins from Arabidopsis and Other Species.

GenBank accession numbers are as follows: *Bos taurus* BtPEX11 (XP_593655); *Canis familiaris* CfPEX11 (XP_545854); *Homo sapiens* HsPEX11α (NP_003838), HsPEX11β (O96011), HsPEX11γ (BAD01558); *Danio rerio* DrPEX11 (XP_694125); *Tetraodon nigroviridis* TnPEX11 (CAG13099); *Saccharomyces cerevisiae* ScPEX11 (NP_014494), ScPEX25 (NP_015213), ScPEX27 (NP_014836); *Aspergillus fumigatus* AfPEX11 (EAL88627); *Aspergillus nidulans* AnPEX11 (EAA65086); *Magnaporthe grisea* MgPEX11 (AAX07688); *Neurospora crassa* NcPEX11 (EAA31192); *Yarrowia lipolytica* YlPEX11 (CAG81724); *Schizosaccharomyces pombe* SpPEX11 (T37974); *Arabidopsis thaliana* AtPEX11a (NP_564514), AtPEX11b (NP_190327), AtPEX11c (NP_563636), AtPEX11d (NP_850441), AtPEX11e (NP_191666); *Oryza sativa* OsPEX11-1 (BAD67925), OsPEX11-2 (CAD41517); *Lycopersicon esculentum* LePEX11 (AAF75750). Sequences were obtained by blasting each of the Arabidopsis PEX11 sequences using the NCBI BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment was constructed using the Clustal W method within the Megalign program from DNASTAR. Bootstrap values, confirming the validity of branches within the alignment, are indicated as percent likelihood of branch accuracy. Bootstrap analysis and neighbor-joining tree were conducted using PAUP* 4.0 software (Sinauer Associates).

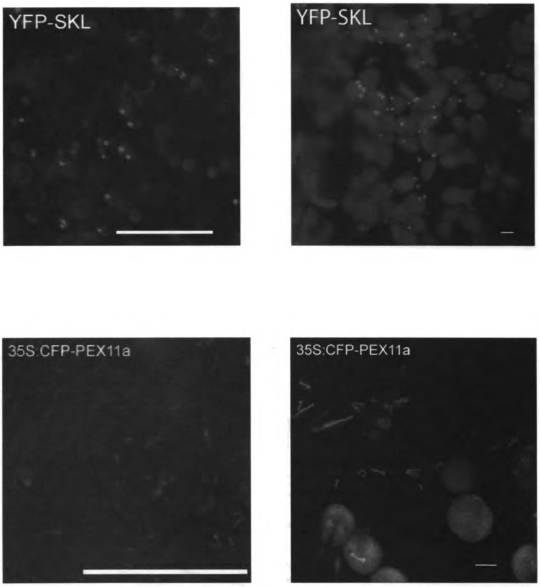
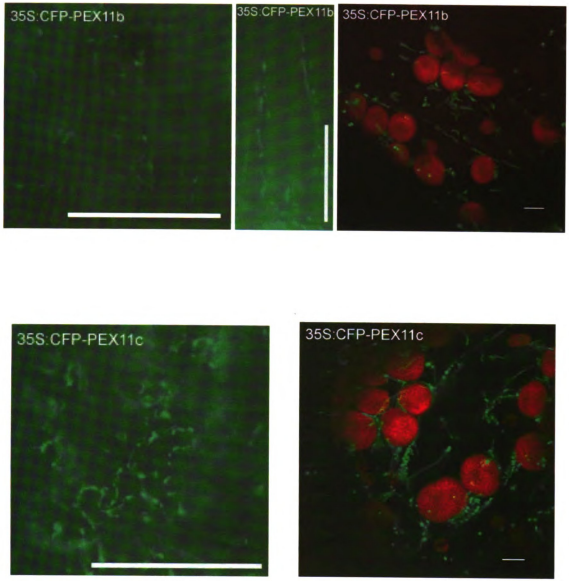


Figure 2.4. Peroxisome Phenotype Conferred by Overexpressing *PEX11* Genes in Arabidopsis.



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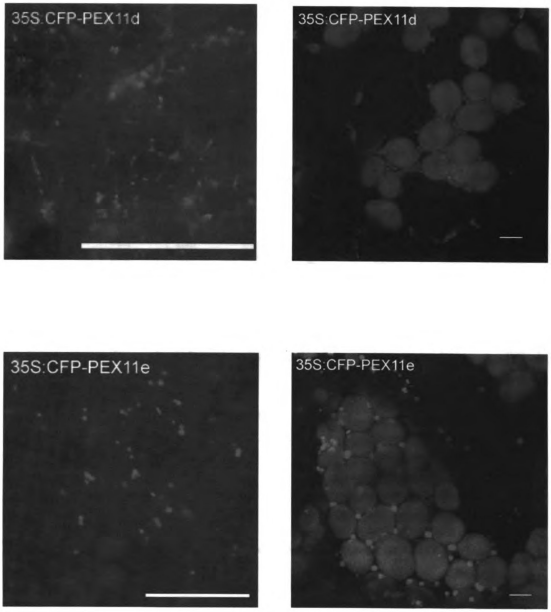

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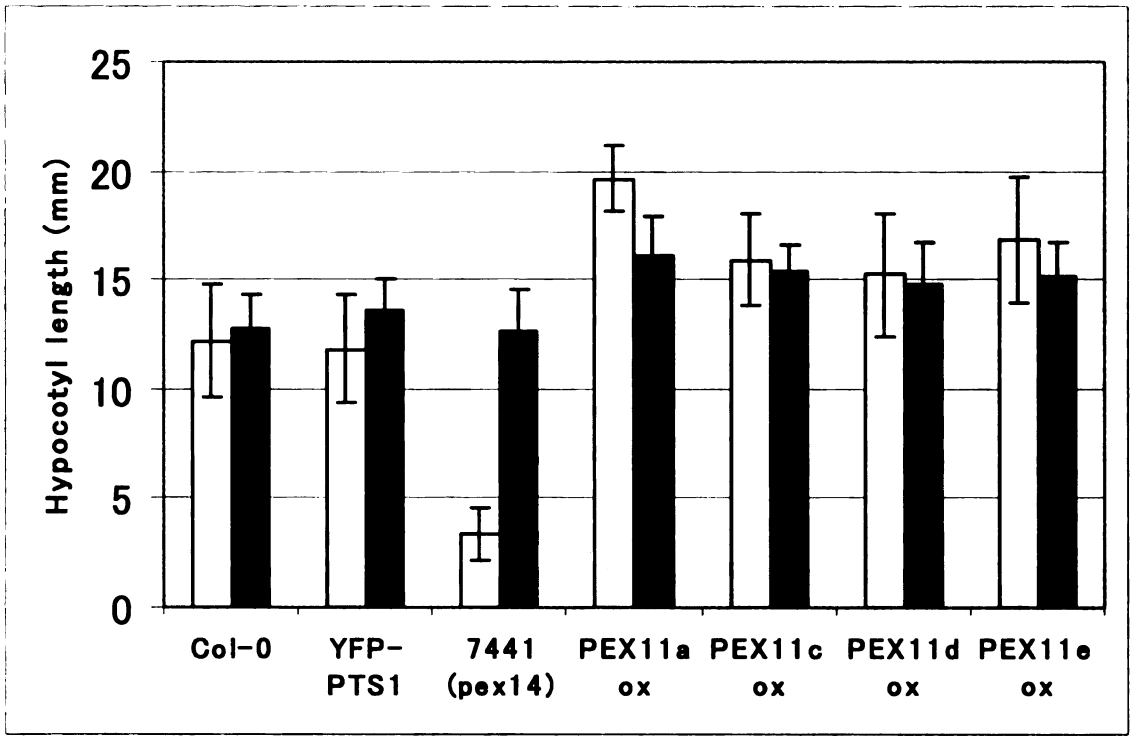

Figure 2.5. Sucrose-dependence Assay of 35S:CFP-PEX11 Lines.

The hypocotyl length of etiolated plants grown on MS media plates in the absence or presence of supplemented 1% sucrose is indicated by the white and grey bars, respectively. Error bars indicate the standard deviations ($n > 34$). Student's T-test determined that the difference in hypocotyl length seen between the YFP-PTS1 and the PEX11 overexpressing lines grown on un-supplemented media was significant to $p = 0.005$.

supplemental sugar due to lack of energy available to these mutants. When Arabidopsis plants are germinated in the presence or absence of sucrose in the dark, hypocotyl lengths can be measured to assess proper peroxisomal functioning (Zolman et al., 2001). Although the *PEX11*-overexpressing lines had apparently normal growth in soil or sucrose-supplemented medium, they had slightly longer hypocotyls than the Col-0 WT and YFP-PTS1 control plants when grown in the absence of sucrose (Figure 2.5). In contrast, the *pex14* knockout mutant (C. Awai and J Hu, unpublished data) of Arabidopsis displayed a significantly reduced level of hypocotyl elongation on the un-supplemented media compared to the 1% sucrose supplemented media, verifying that the assay was working correctly. This result indicates that the peroxisomes in the PEX11-overexpressing lines contain stronger peroxisomal function, possibly due to an increase in the overall volume of peroxisomal matrix due to the increased length and number of peroxisomes compared to control plants. This assay was not conducted on the 35S:CFP-PEX11b transgenic line, due to the lack of a homozygous plant for any of the PEX11b-overexpressing lines at the time of these measurements.

Tissue Specific Expression Analysis of the PEX11 Genes

Expression of each *PEX11* gene was profiled using GENEVESTIGATOR, an Arabidopsis microarray gene expression database and analysis toolbox (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004). This resource uses acquired publicly available microarray data to create an expression profile of the gene being investigated based on the tissues selected. In this analysis the expression levels in seedling, root, stem, cauline leaf, flower, rosette leaf, and seed tissue was examined.

Figure 2.6. Expression Patterns of the *PEX11* Genes in Arabidopsis.

The y axis indicates the level of gene expression in various plant organs. Expression is displayed as a signal expression value assigned by GENEVESITGATOR. Data used for the analysis were retrieved from GENEVESTIGATOR (https://www.genevestigator.ethz.ch; Zimmerman et al., 2004).

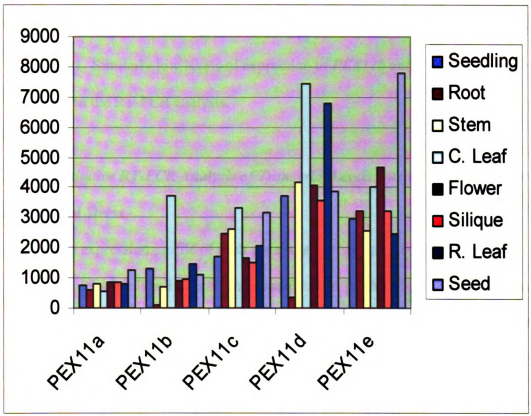

Figure 2.6. Expression Patterns of the *PEX11* Genes in Arabidopsis.

Each *PEX11* gene was expressed in all tissues, with the lowest levels of expression seen for *PEX11*b and *PEX11*d in root tissue, and high levels of expression of these same genes in leaf tissue (Figure 2.6). *PEX11*e also had a high level of expression in seeds. The microarray data were supported by a semiquantitative RT-PCR analysis of each transcript in 10-d seedlings, 20-d seedlings, roots, stems, cauline leaves, flowers, and siliques (Figure 2.7). Similarly low levels of expression of *PEX11b* and *PEX11d* were seen in the root tissue in the RT-PCR analysis.

Semiquantitative RT-PCR Analysis of Transcript Levels During Environmental Stimuli and Stress

The expression profile of each gene was further assessed during different environmental stimuli and stress. The condition that produced the greatest amount of change in any of the transcript levels was a dark-to-light transition of 6-d etiolated seedlings (Figure 2.8). The *PEX11b* transcript level was upregulated during numerous biological replicates of this treatment. No other transcripts showed a consistent up- or down-regulation during this dark-to-light transition. Another condition that produced a consistent upregulation of transcript levels was induced senescence (Figure 2.8). In the 2-d induced senescence treatment all transcript levels were upregulated to some degree, and this up-regulation was again seen in some of the transcripts with the 4-d induced senescence treatment. The upregulation of transcript levels during induced senescence was confirmed by an analogous upregulation seen during natural senescence of rosette leaves (Figure 2.9). The treatments of high light and hydrogen peroxide exposure did not

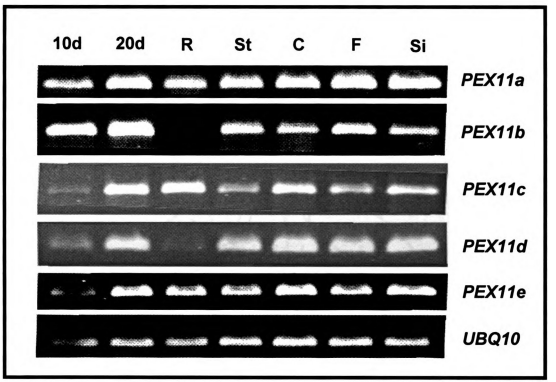

Figure 2.7. RT-PCR Analysis of the *PEX11* genes in Arabidopsis.

Gene-specific primers amplified cDNAs obtained from total RNA extracted from 10-d seedlings, 20-d seedlings, roots (R), stems (St), cauline leaves (C), flowers (F), and siliques (Si). No change is seen in the *UBQ10* transcript, which was used as a loading control. The same number of PCR cycles, 27, was conducted for each reaction.

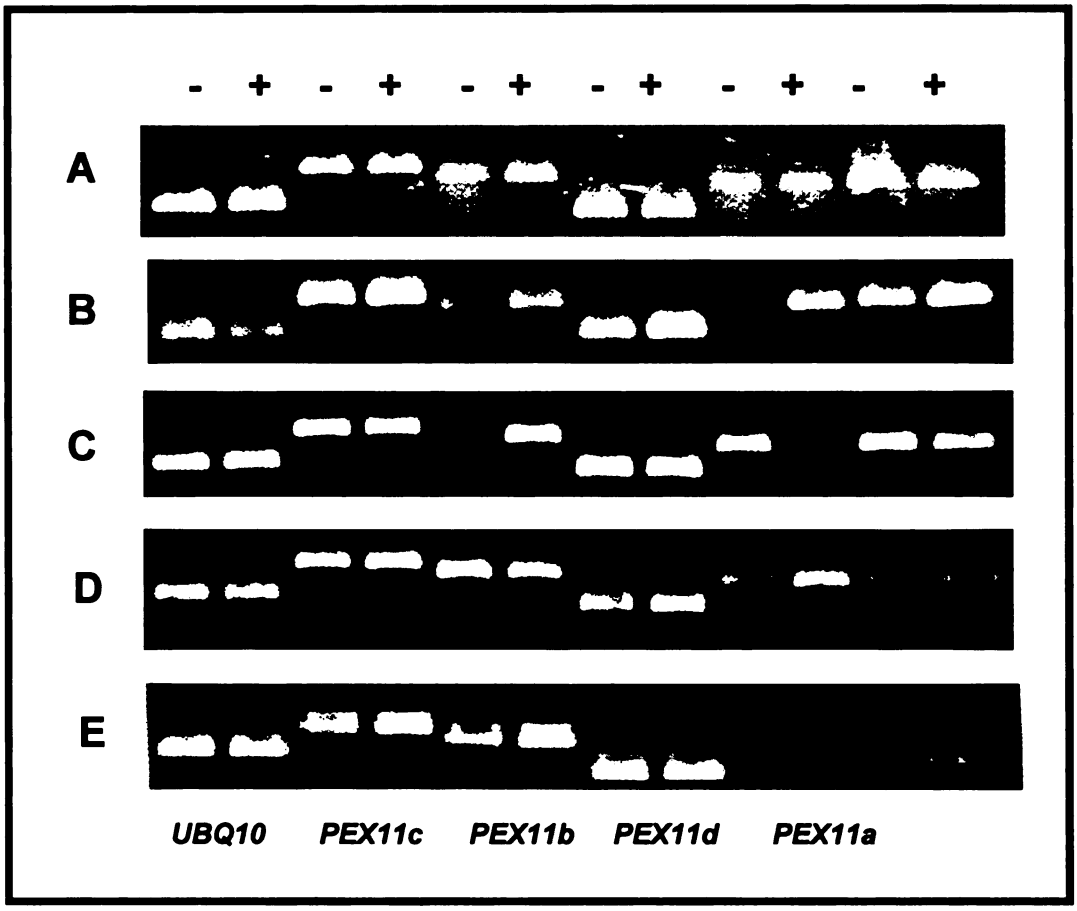

Figure 2.8. Semiquantitative RT-PCR Analysis of *PEX11* Transcript Levels Under Various Conditions.

Total RNA was extracted to conduct RT-PCR reactions from plants subjected to different environmental stimuli such as: dark to light transition (A), for induced senescence 2-d (B), for induced senescence 4-d (C), for hydrogen peroxide exposure 3 hours (D), and for high light treatment 4 hours(E). Lanes are indicated as untreated (-) and treatment (+) samples after 27 PCR cycles. No change is seen in the *UBQ10* transcript, which was used as a loading control.

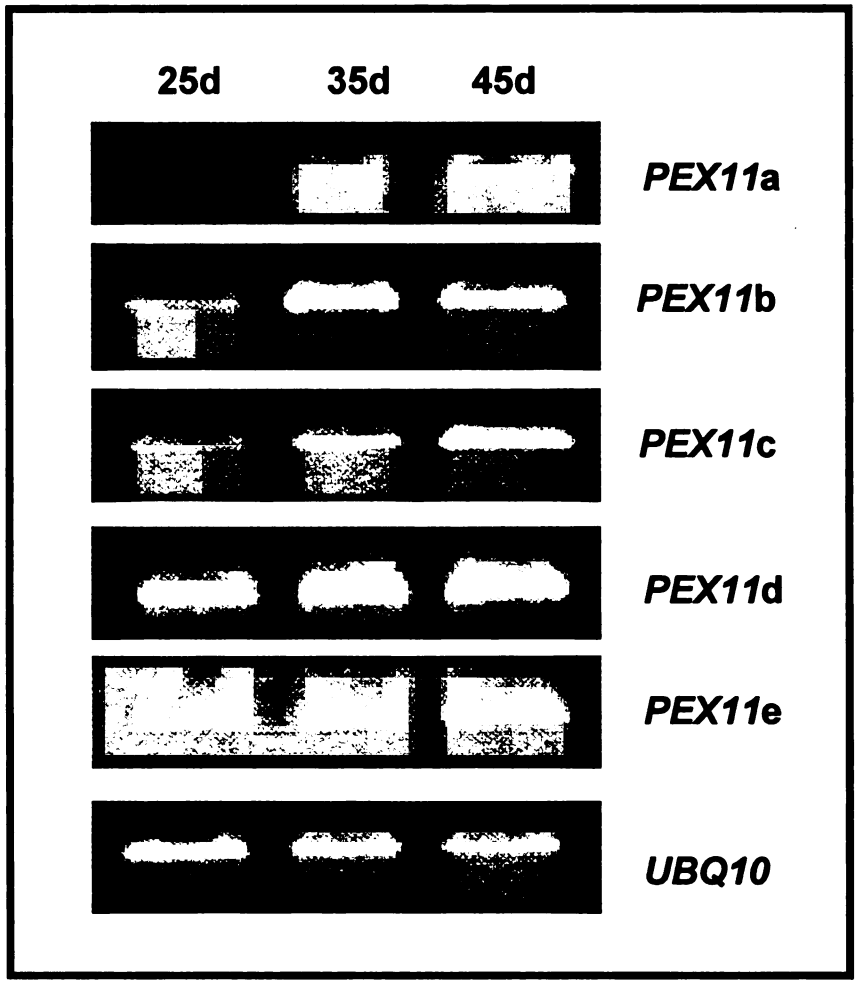

Figure 2.9. *PEX11* Expression During Natural Senescence.

Semiquantitative RT-PCR analysis of the *PEX11* transcript levels from total RNA extracted from rosette leaves of the corresponding ages. *UBQ10* transcript levels were used as a loading control in theses RNA samples.

show consistent upregulation in *PEX11* transcripts. However, during some high light and hydrogen peroxide treatments an upregulation was seen in *PEX11b, PEX11d,* and *PEX11a* (Figure 2.7). Treatments that produced no discernable change in transcript levels in any of the genes include cold, high salt, and low CO_2 (data not shown).

The comprehensive systems-biology database (DSB.DB) (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath/ath_tsgq.html; Steinhauser et al., 2004) was used to examine the types of genes that are co-expressed with the *PEX11* genes. Using this online tool it was found that *PEX11b* and *PEX11d* had the highest level of co-regulation with photosynthetic genes and that *PEX11e* was co-regulated mainly with many genes involved with lipid metabolism (Figure 2.10). This analysis also found that there was a high proportion of genes involved with vesicle fusion that are co-regulated with *PEX11c* (data not shown).

Yeast Two-Hybrid Analysis of PEX11 Protein-Protein Interaction

Studies using the yeast two-hybrid system have shown that the yeast PEX11 protein can form homodimers (Tam et al., 2003). To examine the possibilities that a similar interaction is occurring in with the Arabidopsis PEX11 proteins, a yeast two-hybrid experiment was conducted to detect possible protein-protein interactions between PEX11 family members of Arabidopsis. Test for homodimerization was done for all of the family members and test for heterodimerization was conducted for PEX11a and PEX11b with the other family members. The control proteins pLexA-53 and pB42AD-T were also tested for interaction, which resulted in robust blue colonies, indicating that these two proteins were able to interact and the system was working properly (Table 2.1).

Figure 2.10. DSB.DB Co-Response Database Results for *PEX11d* and *PEX11e*.

Genes that are co-expressed with *PEX11d* and *PEX11e* are displayed as a pie chart with groups of co-expressed genes with similar functions represented by sections of the pie chart that are numbered. These numbered sections account for a percentage of the total number of genes co-expressed with the respective *PEX11* gene. These results were obtained using the DSB.DB (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor.ath/ath_tsgq.html). Pie chart sections are represented as follows:

1 – Photosynthesis
7 – Oxidative pentose phosphate pathway
11 – Lipid metabolism
13 – Amino acid metabolism
19 – Tetrapyrrole synthesis
25 – C1 Metabolism
33 - Development

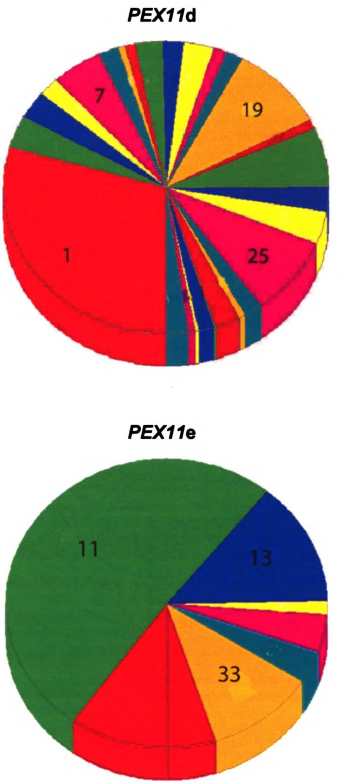

Figure 2.10. DSB.DB Co-Response Database Results for *PEX11d* and *PEX11e*.

No other protein combination resulted in blue colonies on the selective media plates (Table 2.1). To ensure that the lack of interaction was not due to the lack of expression, Western analysis on expression levels of the Arabidopsis PEX11 proteins within the yeast strains was conducted. As shown in Figure 2.11, ample AtPEX11 protein was expressed in yeast. We conclude that the Arabidopsis PEX11 proteins do not interact or that they are not targeted to the nucleus for proper interaction in this assay system.

Discussion

Confirmation that each of the *Arabidopsis thaliana* PEX11 proteins is localized to the peroxisome was accomplished in this study. The localization to the peroxisome is consistent with the localization of PEX11 in other systems such as yeast and mammals (Marshall et al., 1995; Scharader et al., 1998). The method in which PEX11 is directed and incorporated into the peroxisome is not yet clear. However, in yeast and mammalian studies indicate that the PEX11 proteins contain two transmemberane regions with their N- and C-terminal ends facing the cytosol (Abe et al., 1998; Tanaka et al., 2003). Different programs that predict integral membrane spanning regions, such as TMpred (Hofmann and Stofell, 1993), TMHMM (Möller et al., 2001), HMMTOP (Tusnády and Simon, 1998), have also predicted that all of the Arabidopsis PEX11 family members contain integral membrane spanning regions, but the location and number of these regions seems to vary depending on the program utilized (data not shown). However, an integral membrane database, Aramemnon (http://aramemnon.botanik.uni-koeln.de; Schwacke et al., 2003), which specifically calculates plant consensus sequences using 17 membrane prediction programs , shows that only PEX11b and PEX11d contain integral membrane-spanning regions. Therefore, verifying the orientation of the Arabidopsis

Table 2.1. Test for Yeast Two-Hybrid Interaction Between PEX11 Proteins.

The co-expression of pLexA-53 and pB42AD produced a plethora of blue colonies confirming interaction between these two proteins and proper functioning of the system. No blue colonies were observed with any tested protein combination of AtPEX11s. No blue colonies were observed with the interaction between pLexA53 and pB42AD with plates lacking galactose, which acts as a negative control to validate positive interactions seen on plates with galactose.

Table 2.1. Test for Yeast Two-Hybrid Interaction Between PEX11 Proteins.

pGilda	pB42AD	Plate	Interaction (blue colony)
pLexA-53	pB42AD-T	SD Gal/Raf – UHTL	+
pLexA-53	pB42AD-T	SD Glucose – UHTL	-
PEX11a	PEX11a	SD Gal/Raf – UHTL	-
PEX11b	PEX11b	SD Gal/Raf – UHTL	-
PEX11c	PEX11c	SD Gal/Raf – UHTL	-
PEX11d	PEX11d	SD Gal/Raf – UHTL	-
PEX11e	PEX11e	SD Gal/Raf –UHTL	-
PEX11a	PEX11b	SD Gal/Raf – UHTL	-
PEX11a	PEX11c	SD Gal/Raf – UHTL	-
PEX11a	PEX11d	SD Gal/Raf – UHTL	-
PEX11a	PEX11e	SD Gal/Raf – UHTL	-
PEX11b	PEX11a	SD Gal/Raf –UHTL	-
PEX11b	PEX11c	SD Gal/Raf – UHTL	-
PEX11b	PEX11d	SD Gal/Raf – UHTL	-
PEX11b	PEX11e	SD Gal/Raf – UHTL	-

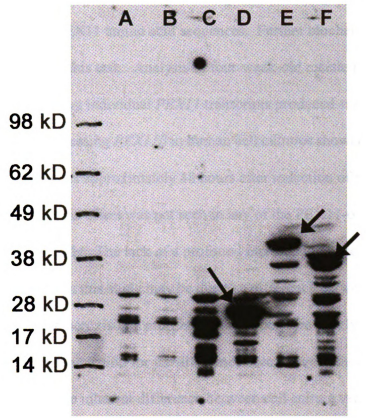

Figure 2.11. Western Analysis of Protein Expression in Yeast.

Expression of HA-tagged proteins is visualized using anit-HA antibody from total protein from extracted from the following yeast strains: EGY48 p[80p-lacZ] (A), pGilda empty vector (B), pGilda + pB42AD empty vector (C), pGilda + pB42AD PEX2-RING Finger Domain (D), pGilda PEX11a + pB42AD PEX11a (E), and pGilda PEX11d + pB42AD PEX11d (F). Bands indicating the presence of HA-tagged proteins are observed in the PEX2-RF ~28kD, PEX11a ~43kD, and PEX11d ~38kD samples. Arrows indicate predicted HA-tag detection.

PEX11 proteins within peroxisomal membranes cannot be accomplished by merely *in silico* analysis of the AtPEX11 amino acid sequences. Further biochemical analysis need to be done to accomplish this task. Analysis of four-week-old rosette leaves from Arabidopsis overexpressing individual *PEX11* transcripts produced surprising results. Previous studies overexpressing *PEX11β* in human cell cultures showed that cells became saturated with peroxisomes approximately 48 hours after induction of gene expression (Li and Gould, 2002). This effect was not seen in any of the PEX11-overexpressing plant lines examined in this study. The lack of a profound increase in peroxisome numbers in the overexpression lines in this study may be due to a difference between the mammalian and plant systems where peroxisome proliferation may be more tightly regulated in plants. Another likely explanation for the difference seen between these two systems/experiments is the inherent difference between cell culture versus whole-organism studies. A third possibility is that the function of PEX11 in plants may have diverged from that of mammals in that several of the Arabidopsis PEX11 proteins, acting in concert, are needed to exert dramatic proliferation effects. To examine this possibility, several PEX11 proteins would need to be overexpressed together in the future.

Although a drastic increase of peroxisome numbers was not seen in the *PEX11*-overexpressing lines, altered peroxisomal morphology was observed. The different peroxisomal morphology phenotypes observed with overexpressing lines provide an indication to the function of each gene being overexpressed. The extreme elongation of peroxisomes seen in plants overexpressing *PEX11a* and *PEX11b* indicates that these genes may be involved primarily in the initial membrane expansion/organelle elongation before further division and subsequent separation of peroxisomes occur. A similar type

of elongation was seen transiently in human tissue culture cells overexpressing *PEX11β* (Li and Gould, 2002; Schrader et al., 1998). In these experiments *PEX11β*-induced elongation preceded a drastic increase in peroxisome numbers. Overexpression of *PEX11*c in Arabidopsis resulted in both elongated peroxisomes and clustered peroxisomes. This clustering was also observed in *PEX11*d-overexpressing plants. The cause of the clustering effect in the PEX11c and PEX11d overexpression plants is not clear. Under the certain conditions, it has been shown that GFP proteins form homodimers in a non-specific manner, making them amenable to forming large aggregates (Hofmann et al., 2002). The attachment of CFP to the N-terminal end of each of the PEX11 proteins could in theory result in the CFP moieties forming aggregates. However, similar results were obtained in plant overexpressing PEX11 alone, making this hypothesis less reliable (data not shown). An alternative explanation for the clustering in 35S:CFP-PEX11c and 35S:CFP-PEX11d plants is that these proteins are merely involved in initiation, not completion, of the fission process. The actual separation machinery may not be able to keep up with the rate of desired peroxisome proliferation evoked by overexpressing specific PEX11 proteins. Therefore clusters of peroxisomes possibly develop from the lack of synergy between the two hypothetical steps of peroxisome proliferation.

The slightly enlarged peroxisomes seen in *PEX11*e-overexpressing plants are not seen in plants overexpressing of any of the other *PEX11* genes. This unique phenotype supports the hypothesis that each of the *PEX11* genes in Arabidopsis may play a unique and non-redundant role in peroxisome proliferation. Given the sequence divergence and different overexpression peroxisomal phenotypes, our working model is that PEX11a and

PEX11b are responsible for elongation of the peroxisome membrane and that PEX11c, PEX11d, and possibly PEX11e may be involved in both membrane growth and constriction. The dynamin-related protein, DRP3A, may participate in the latter steps of peroxisome division by powering the separation of the membranes (Figure 2.12).

The effect on the physiological function of the severely altered peroxisomal morphology seen in the PEX11-overexpression lines was analyzed using the sucrose assay. During germination of oilseed species, peroxisomes use β-oxidation and the glyoxylate cycle to aid in converting stored lipids and fatty acids to sugars, which can then be used to facilitate proper plant growth and development. Plants containing dysfunctional peroxisomes that cannot efficiently facilitate the β-oxidation reactions and the glyoxylate cycle display poor growth in the absence of sucrose (Zolman et al., 2001). The increased length of hypocotyls seen in the PEX11-overexpressing lines is correlated with peroxisomal elongation in 35S:CFP-PEX11a and 35S:CFP-PEX11b and increased numbers in the 35S:CFP-PEX11c, 35S:CFP-PEX11d, and possibly 35S:CFP-PEX11e lines. The increase in hypocotyl length seen in all transgenic lines examined may be due to an increase in overall volume of peroxisome matrix even though this increase is not easily discernable in some lines. These PEX11-overexpressing plants were phenotypically identical to Col-0 WT plants when grown in the soil (data not shown), again indicating that the peroxisomes were fully functional within these plants.

The analysis of the tissue specific expression pattern of the *PEX11* genes in different plant organs found that *PEX11b* and *PEX11d* were expressed at extremely low levels in roots and expressed at greatly increased levels in cauline and rosette leaves. This expression profile suggests that these two genes are involved primarily in

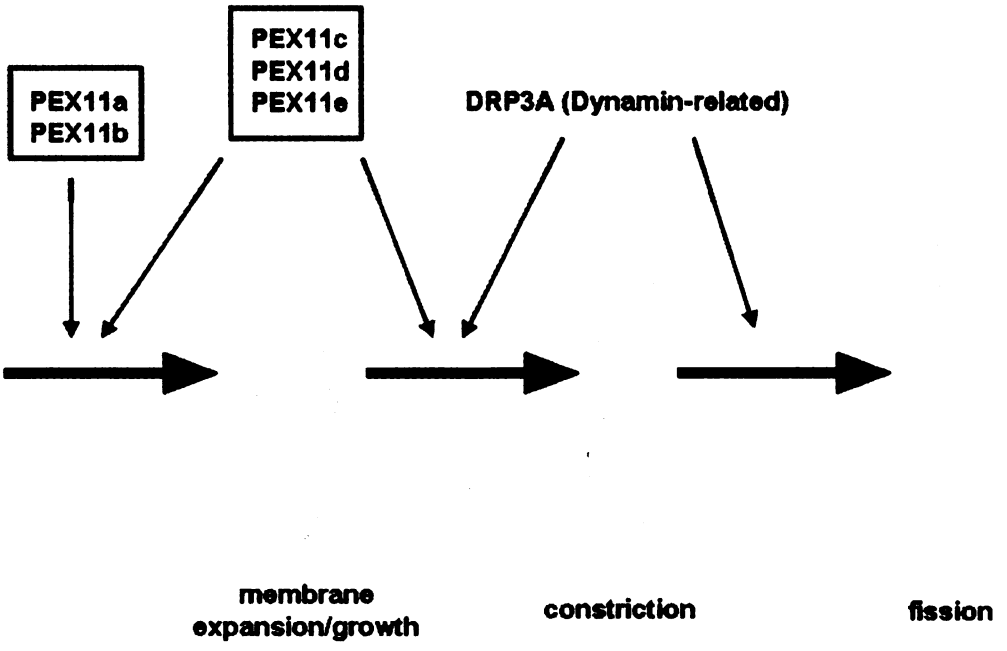

Figure 2.12. A Working Model for Peroxisome Proliferation in Arabidopsis.

This model (see text for details) was constructed based on the observations from this study of PEX11s and based on previous work of the DRP3A protein (Mano et al., 2004).

influencing peroxisome proliferation in photosynthetic tissue. Examination of the expression levels of these genes in a dark to light transition has also shown that *PEX11b* is strongly induced by light exposure. Analysis of the *PEX11*b promoter shows that it contains an increased number of putative light responsive elements (M Desai and J Hu; unpublished results). Together these results suggest that *PEX11b* is responsive to light and might play a role in conjunction with *PEX11d* in promoting peroxisome proliferation in response to light, including the transition of glyoxysomes to leaf peroxisomes. Moreover, *PEX11e* had an very high expression level in seeds, suggesting that this gene could play a major role in the proliferation of glyoxysomes. Results in the comprehensive systems-biology database (DSB.DB) (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath/ath_tsgq.html; Steinhauser et al., 2004) are consistent with the tissue specific expression patterns determined by RT-PCR in this study (Figure 2.7). For example, *PEX11b* (data not shown) and *PEX11d* had the highest level of co-regulation with photosynthetic genes and that *PEX11e* was co-regulated mainly with many genes involved with lipid metabolism (Figure 2.10).

Analysis of the *PEX11* transcript levels during different environmental stimuli and stresses revealed that transcript levels of many *PEX11* genes were responsive to other stimuli in addition to the dark to light transition. Induced senescence produced the greatest response in transcript levels, with an upregulation consistently seen in all *PEX11* transcripts. Plant senescence is the process of recycling nutrients that can be used in younger newly developing plant tissues; however, how this process is regulated in plants is still largely unknown (Lin and Wu 2004; Yoshida, 2003). Previous studies have shown that peroxisomes are possibly involved in plant senescence, with roles in membrane lipid

catabolism into carbohydrate, nitric oxide signaling, and the proteolytic cleavage of proteins (Corpas et al., 2004; Distefano et al., 1999; Distefano et al., 1997). The second messenger, nitric oxide, allows plants to systematically coordinate the degradation of proteins by proteolytic cleavage to be reused in newly formed tissues. Although a noticeable increase in peroxisome numbers was not observed in older leaf tissue (data not shown), an overall increase in transcript levels was observed by each of the *PEX11* genes as plant tissue aged (Figure 2.9). Assuming that the Arabidopsis PEX11 proteins have a direct role in peroxisome proliferation, the increase in *PEX11* transcript levels during induced senescence and natural plant aging is consistent with previous research that have suggested that peroxisomes are involved with the process of plant senescence (Pastori and del Río, 1997). However, the increase in transcript level seen during induced and natural senescence was not nearly as great as that observed when controlled by the 35S promoter (data not shown), which may be the reason why a peroxisomal phenotype is observed in the transgenic overexpressing plants and not in the senescing tissue.

The mechanism governing peroxisome proliferation in plants as well as in yeast and mammals is still largely unknown. However, previous studies in yeast have shown that a multitude of proteins are involved in the process, possibly by forming complexes to initiate the division process (Hoepfner et al., 2001; Koch et al., 2004; Li and Gould, 2003; Mano et al., 2004; Vizeacoumar et al., 2004). Previous yeast two-hybrid analysis showed that the yeast PEX11 protein is able to form homodimers (Marshall et al., 1996; Rottensteiner et al., 2003; Tam et al., 2003). However, we have found that none of the Arabidopsis PEX11s are able to form homodimers using the yeast two-hybrid technique. This result does not seem to be a problem with the system or protein expression. A

simple explanation is that the Arabidopsis PEX11 proteins may simply not be able to form oligomers as seen in the yeast system. Alternatively, the localization of the Arabidopsis PEX11 proteins to peroxisomes may have prevented these proteins from targeting to the yeast nucleus where they would be functional for this method of protein interaction detection. A different method of protein interaction detection, such as the mating-based split ubiquitin system (Obrdlik et al., 2004) or the fluorescence resonance energy transfer technique (Gordon et al., 1998), need to be implemented to possibly detect interactions between the Arabidopsis PEX11 proteins.

Although the exact mechanistic process of peroxisome division and proliferation is not clear, it is evident that other proteins, in addition to the PEX11 proteins, play a role in this process. In both yeast and mammals dynamin-related proteins have been shown to be intimately involved in the fission process of peroxisomes (Li and Gould, 2003; Hoepfner et al., 2001). Recently, work on DRP3A has verified that dynamin-related proteins are also involved with peroxisome proliferation in plants (Mano et al., 2004). The association and synergy between the dynamin-related proteins and the PEX11 family of proteins in Arabidopsis has not yet been addressed; the interplay between the dynamin-related proteins and the PEX11 proteins could reveal interesting aspects of peroxisome proliferation in plants.

Further analysis of loss-of-function mutants, such as PEX11 knock-out or RNAi plants, as well as protein interaction studies need to be done to further elucidate the role for this protein family in peroxisome proliferation in Arabidopsis. Identifying players in peroxisome proliferation and mechanisms underlying the regulation of this process in plants during different developmental as well as environmental changes is very important

to the field of plant biology, due to the essential nature of peroxisomes. Our work will contribute to the understanding of how this vital organelle functions and proliferates in the plant.

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CHAPTER 3

Virus-Induced Gene Silencing and Tissue Specific Expression Analysis of the *Arabidopsis thaliana PEX12* Gene

Abstract

The import of proteins into the peroxisomal matrix is a highly regulated and complex process facilitated by a number of peroxins encoded by the *PEX* genes. Specifically, the three RING finger peroxins, PEX2, PEX10, and PEX12, are believed to be essential components of the protein import apparatus in yeast and mammals. To determine the role for PEX12 in peroxisome biogenesis and development in plants, we used the virus-induced gene silencing technique to reduce the expression of *PEX12* in Arabidopsis. We showed that the number of peroxisomes and import of matrix proteins were both decreased when PEX12 was silenced. Analysis of the expression pattern of the *PEX12* gene revealed that the three RING peroxin genes are mostly co-expressed throughout Arabidopsis development. This study substantiates the role for PEX12 in peroxisome biogenesis and supports the notion that the three RING finger peroxins function in concert.

Among the over 30 PEX proteins identified so far, PEX2, PEX10, and PEX12 RING finger proteins are believed to act in conjunction to facilitate the import of cargo proteins and the recycling of receptors in yeast and mammals, and possibly in other eukaryotes. These three peroxins are integral membrane proteins whose N- and C-terminal domains are both predicted to be exposed to the cytosol, yet the process in which they mediate matrix protein import and PEX5 receptor recycling remains largely enigmatic (Purdue and Lazarow, 2001; Brown and Baker, 2003). Additionally, a complex array of interactions involving at least 10 PEX proteins seems to take place during matrix protein import and subsequent receptor recycling (Ange et al., 2003; Collins et al., 2000; Eckert and Johnsson, 2003). The understanding of the complete functions of each PEX protein individually as well as within a complex is still very tentative. The essential nature of these *PEX* genes in plants, however, is exhibited in the embryonic lethal phenotype displayed in null mutants of *PEX2*, *PEX10*, and *PEX12* (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005).

Mutations in PEX12 lead to failure of matrix protein import in yeast and mammals and result in the Zellweger syndrome, a lethal neurological disorder in humans (Gould and Valle, 2000). A PEX12-CFP protein expressed in a background YFP-PTS1 plant has confirmed the localization of PEX12 to the peroxisome (Fan et al., 2005). Overexpression of the *PEX12* transcript produced no discernable phenotype within the peroxisome morphology or in the whole plant of Arabidopsis (Fan and Hu, unpublished data). The embryo-lethal phenotype of the *pex*12 knockout plants prevented us from further elucidating the potential roles of PEX12 in peroxisome biogenesis and in later

stages of development (Fan et al., 2005); thus, mutants with reduced levels of PEX12 were needed.

A gene-silencing system based on the bipartite geminivirus cabbage leaf curl virus (CbLCV) was recently developed that can efficiently induce diffusible, homology-based systemic silencing of endogenous genes in Arabidopsis (Turnage et al., 2002; Robertson, 2004). This system is composed of two small circular viral DNA genomes: CbLCV A and CbLCV B. To attenuate the viral symptom, the coat protein-encoding *AR1* gene was deleted from the A genome and replaced by a fragment of the gene to be silenced. The B genome carries the movement protein for systemic infection (Turnage et al., 2002).

To address the role of PEX12 in plant cellular functions and to illustrate its role in plant development, partial characterization of the Arabidopsis PEX12 protein was conducted using virus-induced gene silencing along with tissue specific expression profiling of the *PEX12* transcript. This work establishes an essential role for Arabidopsis PEX12 in peroxisome biogenesis and reveals the similarity of expression profiles between *PEX12* the other two Arabidopsis RING peroxin-encoding genes, *PEX2* and *PEX10*.

Material and Methods

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants used in this study are of the Columbia (Col-0) background. Seeds were germinated on 1X Murashige and Skoog medium (Gibco). Plants used for the expression analysis were grown with 16/8-h light/dark

photoperiod under 80 to 100 μmol m^{-2} s^{-1} light conditions at 22°C. Plants used for bombardment in virus-induced gene silencing were grown in the same light intensity and temperature in short-day conditions with an 8/16-h light/dark photoperiod.

Epi-Fluorescence Microscopy

A Zeiss Axiophot microscope was used to visualize fluorescent proteins. For in vivo detection of YFP, leaf tissue was mounted in water and viewed with a YFP filter (excitation 500 ± 12.5 nm, emission 540 ± 20 nm).

Virus-Induced Gene Silencing

Arabidopsis plants in the YFP-PTS1 background were grown in individual pots in short-day conditions and bombarded with an equal amount of each silencing construct DNA (in CbLCVA) and the pCPCbLCV.008 DNA (CbLCV B) as described in a previous study (Turnage et al., 2002). Fan et al. (2005) describes the cloning of the PEX12-silencing fragment into pCPCbLCV.007 vector. Each plant was bombarded at the age of 3- to 4-weeks-old, according to the protocol provided by Turnage et al. (2002). Two to three plants were bombarded with each construct. The experiment was repeated three times. "Old" and "new" leaf tissue was collected separately from infected plants approximately 4 weeks after bombardment. "New leaves" were those from around the center of the rosette that emerged after bombardment, and the "old leaves" were older rosette leaves that were present at the time of the bombardment. Because of the distinct colors of silenced and nonsilenced leaves, the CH42-infected plants served as a guide to

distinguish ''new'' from ''old'' tissue for microscopic and reverse transcriptase (RT)-PCR characterization (Figure 3.2).

Images in this thesis are presented in color.

RT-PCR Analysis of PEX12 Transcripts

Total RNA was extracted with TRIzol reagent (Invitrogen) and subjected to reverse transcription reaction (Gibco). The PEX12-specific primers PEX12F2 (5'-GCGAGATTGAGATTGAGGAAAGACAGTGCC-3') from exon 3 and PEX12R (5' GGAGGGTACACTGTTGGAGCTGATAATCTC-3') from exon 8 amplify a 684-bp product from PEX12 cDNA. The ubiquitin-specific primers UBQ10-1 (5' TCAATTCTCTCTACCGTGATCAAGATGCA-3') and UBQ10-2 (5' GGTGTCAGAACTCTCCACCTCAAGAGTA-3') from the *UBIQUITIN10* gene (At4g05320) were used to amplify a cDNA product of approximately 320 bp. PCR conditions were as follows: 94°C for 3 min, followed by cycles (27 for Figure 3.3 and 36 for Figure 3.4) of 94°C for 45 s, 57°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 7 min.

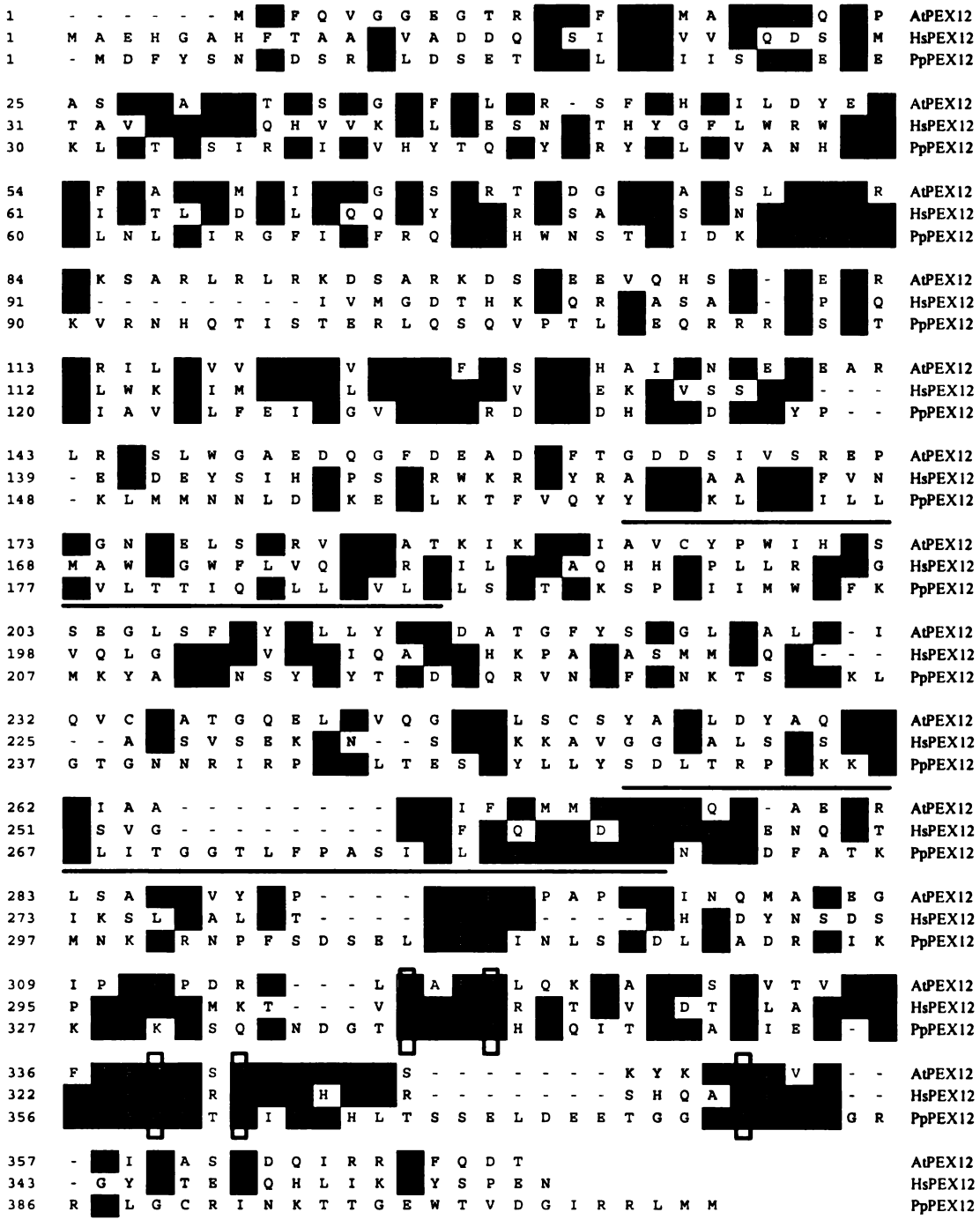
Results

Amino Acid Sequence Analysis of PEX12

AtPEX12 (At3g04460) is a single-copy gene encoding a putative protein of 44 kD. It shares approximately 27% protein sequence identity with its yeast and mammalian orthologs and contains a C_5-type RING finger motif with five conserved Cys (Figure

Figure 3.1. Amino Acid Sequence Alignment of PEX12 Proteins.

Arabidopsis AtPEX12 (Q9M841); *Homo sapiens* HsPEX12 (O00623); *Pichia pastoris* PpPEX12 (Q01961). Sequences were aligned using Megalign from DNASTAR. Underlined are putative transmembrane domains. Boxes indicate the conserved Cys residues in the C-terminal RING finger motif.



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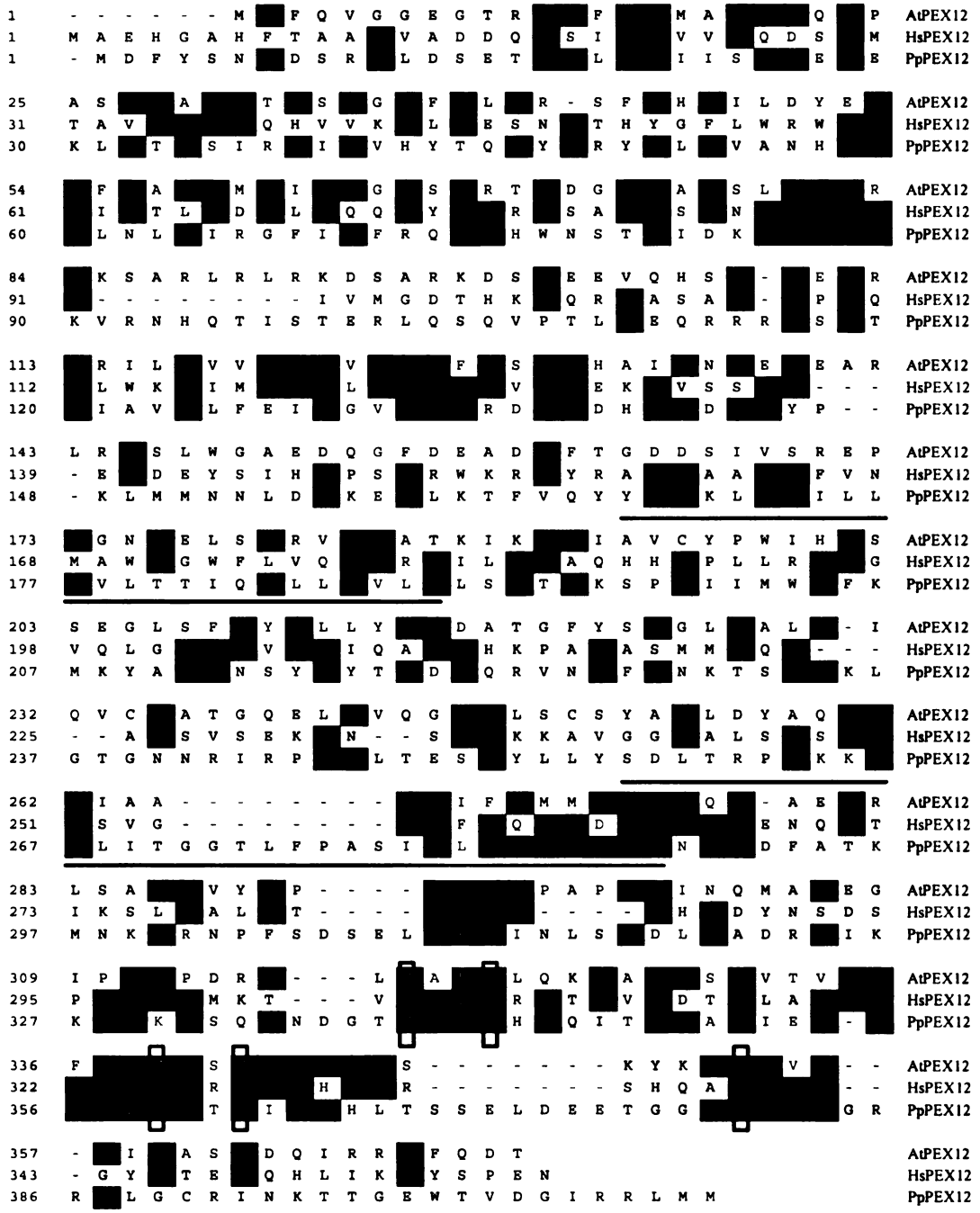

Figure 3.1. Amino Acid Sequence Alignment of PEX12 Proteins.

3.1), which is different from the C_3HC_4-type RING found in AtPEX2 and AtPEX10. Two putative transmembrane regions are also predicted to anchor the PEX12 protein in the peroxisomal membrane with the N- and C-termini facing the cytosol (Figure 3.1).

Virus-Induced Gene Silencing of PEX12

To silence the *PEX12* gene, a 247-bp cDNA fragment of *PEX12* was cloned into the CbLCV A vector in the sense or antisense orientation (Fan et al., 2005). Viruses containing the silencing constructs were bombarded into Arabidopsis YFP-PTS1 plants. Leaf tissue from infected plants was observed under the fluorescent microscope 3 to 4 weeks after bombardment, when genes encoded by the viruses were expressed at high levels in new leaves. As a control, we also bombarded some plants with viruses containing the *CHLORATA42* (*CH42*) gene. *CH42* encodes the small subunit of the chloroplast magnesium-chelatase (Koncz et al., 1990) and confers an albino phenotype when silenced, owing to the lack of chlorophyll production (Figure 3.2, A). This control is used as an indicator for massive viral replication and systemic movement and therefore serves as a guide to determine the time for RNA and fluorescent microscopic analyses.

Plants infected by both the sense and antisense *PEX12*-silencing constructs exhibited a strong reduction in the number of peroxisomes as well as peroxisomal fluorescence of the YFP-PTS1 protein in new leaves (Figure 3.3, C and E) compared to old leaves (Figure 3.3, D and F), whereas plants infected by the empty vector control did not show a significant difference between old and new leaf tissue (Figure 3.3, A and B). RT-PCR analysis was subsequently performed to determine the expression level of *PEX12* in these tissues. Figure 3.3G shows that, in plants bombarded with the *PEX12*-silencing constructs, the transcript level of *PEX12* in the new tissue was significantly

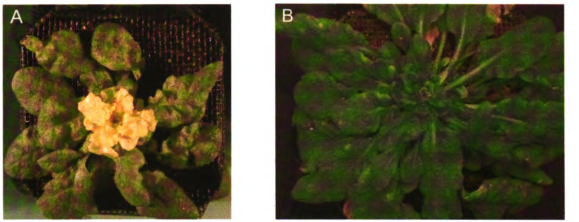

Figure 3.2. Phenotype of Virus-Induced Gene Silencing (VIGS) Plants.

(A) is the control *CH42*-silenced plant. Albino leaves are newly emerged tissue in which the *CH42* gene is silenced. (B) shows a plant infected by a *PEX12*-silencing construct. Both plants display viral symptoms of curled leaves and the lack of proper inflorescence development.

lower than in the old tissue, suggesting that PEX12 is required for peroxisome biogenesis in leaves. Despite the fact that the CbLCV virus used in this work was attenuated by removal of the *AR1* gene, plants still displayed mild viral symptoms after infection, such as wrinkled leaves, stunted growth, and lack of inflorescence (Figure 3.2, B). As such, the mutant phenotypes caused by *PEX12* silencing in adult plants could not be unambiguously determined by this approach.

Expression Profile of AtPEX12

The essential role of PEX12 throughout Arabidopsis development (Fan et al., 2005) led us to examine its expression pattern in the plant. An RT-PCR analysis of the *PEX12* transcript suggested that this gene was expressed in young seedlings, leaves, roots, flowers, and siliques, with a significant increase of transcript seen in flowers and sliques compared to the other tissues analyzed (Figure 3.4). To assess its expression more completely, GENEVESTIGATOR, an Arabidopsis microarray gene expression database and analysis toolbox (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004) were used to search for expression of the Arabidopsis *PEX12* gene in various organs. The microarray data, based on experiments with the Arabidopsis fullgenome chip arrays, showed that *AtPEX12* was ubiquitously expressed and that its expression pattern correlated well with several other genes known to be required for peroxisome biogenesis, including the other two RING peroxin genes, *PEX2* and *PEX10* (Figure 3.5). Consistent with their essential role in embryogenesis, all three RING peroxins were most highly expressed in seeds (Figure 3.5). The transcript levels of these genes were also high during germination (Fan et al., 2005), when peroxisomes are needed for lipid metabolism to

Figure 3.3. Virus-Induced Gene Silencing of *PEX12*.

A to F, YFP-PTS1 fluorescence in plants infected by virus containing the CbLCV empty vector (A and B), a vector containing a fragment of *PEX12* in the sense orientation (C and D), and a vector containing the antisense fragment of *PEX12* (E and F). A, C, and E, leaves from new growth; B, D, and F, old leaves of the same plants. Bars = 10 μm. G, RT-PCR analysis of *PEX12* and *UBIQUITIN10* (*UBQ10*) transcripts. Lanes 1 to 6 are PCR products amplified from RNA from A to F.

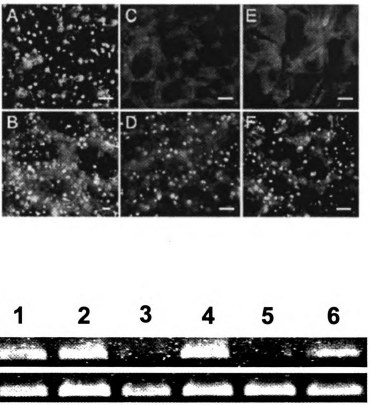

Figure 3.3. Virus-Induced Gene Silencing of *PEX12*.

produce a carbon source for germination, and in senescent plants (Figure 3.5), in which leaf peroxisomes are mostly converted to glyoxysomes to facilitate metabolism of membrane lipids, to stimulate the proteolytic cleavage of plant proteins (Distefano et al., 1999), and to store nitric oxide, a signaling molecule believed to play a role in senescence (Corpas et al., 2004). All three PEX genes were also abundantly expressed in floral structures, including, inflorescences, carpels, and pedicels (Figure 3.5), and at the stage when flowering is complete and siliques are formed (Fan et al., 2005).

Discussion

In this study, we present evidence that PEX12 is an essential protein for proper peroxisomal biogenesis in Arabidopsis and that the *PEX12* transcript displays a parallel expression profile to other Arabidopsis RING peroxins. YFP-PTS1 plants infected by the CbLCV virus carrying part of the *PEX12* coding sequence displayed a strong reduction of the number of peroxisomes and import of PTS1-containing matrix proteins. This data, in addition to the embryo lethal phenotype of null mutant plants, suggests that PEX12, just like PEX2 and PEX10, is a basic component of plant peroxisomes, facilitating proper peroxisomal biogenesis. The role in which reduced *PEX12* transcript levels effects peroxisome biogenesis or peroxisomal matrix protein import is still not completely understood in plants. The greatly-reduced YFP-PTS1 signal in *PEX12* VIGS tissue could be attributed to a lack of peroxisomes for the YFP protein to localize or disrupted import of YFP-PTS1 proteins into peroxisomes (Figure 3.3). Whether the lack of PEX12 causes a direct inhibition of peroxisome biogenesis or if the lack of peroxisomes is a secondary effect due to disrupted peroxisomal matrix protein import has yet to be determined.

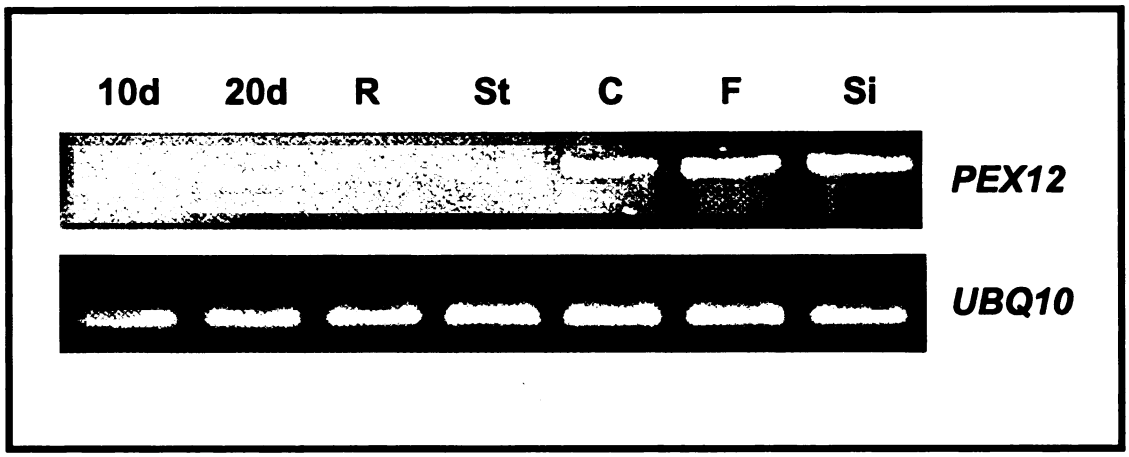

Figure 3.4. RT-PCR Analysis of *PEX12* Transcript Levels in Arabidopsis Tissues. *PEX12*- and *UBQ10*-specific primers amplified cDNA obtained from total RNA extracted from 10-d seedlings, 20-d seedlings, roots (R), stems (St), cauline leaves (C), flowers (F), and siliques (Si).

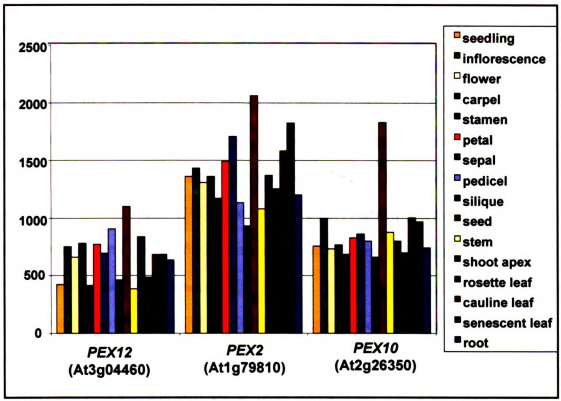

Figure 3.5. Expression Patterns of the RING *PEX* Genes in Arabidopsis.

Expression levels of *PEX* RING finger transcripts in various plant organs. The y axis indicates the level of gene expression displayed as a signal intensity value assigned by GENEVESTIGATOR. Data used for the analysis were retrieved from GENEVESTIGATOR (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004).

Peroxisomal matrix proteins are mislocalized in the cytoplasm in yeast and animal cells with reduced function of PEX12, indicating that this protein is particularly required for protein import into peroxisomes (Chang et al., 1997; Okumoto et al., 2000). Mammalian cells lacking PEX12 also showed accumulation of the PTS1 receptor PEX5 at the cytosolic side of the peroxisome membrane, suggesting that PEX12 may mediate recycling of PEX5 (Dodt and Gould, 1996). Mutations within PEX12 have also been associated with a specific class of peroxisome genetic diseases which display severe symptoms similar to those of Zellweger Syndrome, the most severe peroxisomal genetic disease (Chang et al., 1997; Gootjes et al., 2004). The greatly reduced number of peroxisomes in the *PEX12*-VIGS plants and the embryo lethality in the *PEX12* null mutant plants (Fan et al., 2005) demonstrate the similarities within the phenotypes of both plant and mammalian systems.

A search of the GENEVESTIGATOR microarray database revealed similar expression patterns of the three RING peroxins in some tissues, supporting the essential roles of these *PEX* genes in seed development, germination, flower formation and senescence. The fairly ubiquitous expression pattern displayed by each of the RING *PEX* transcripts in several plant organs supports previous results showing these genes to be essential for proper plant development (Hu et al., 2002; Sparkes et al., 2003; Fan et al., 2005). The high level of expression seen in the reproductive organs is consistent with the finding that flower formation is disrupted when the expression of *PEX12* was strongly reduced in transgenic Arabidopsis plants expressing a *PEX12* double-stranded RNAi (dsRNAi) construct (Fan et al., 2005). The similar expression profile displayed by each of the RING finger peroxins is indicative of the parallel role that each of these proteins

plays in peroxisome biogenesis and more specifically peroxisome matrix protein import. However, the variations seen in the expression profile of these transcripts, such as the high level of *PEX10* expression in seeds (Figure 2.5), with respect to the formation of a three member RING finger complex, is still not entirely known. Additionally, the role that these proteins are playing in tissues such as the shoot apex (Figure 2.5), is also yet to be determined.

The similar phenotypes caused by loss-of-function of each of these three genes and the co-expression pattern in Arabidopsis support the notion that AtPEX2, AtPEX12, and AtPEX10 act closely during peroxisome biogenesis (Baker and Sparkes, 2005). However, it will be necessary to test for interactions among the three RING peroxins and between these proteins and other peroxins in Arabidopsis to elucidate the specific function of each of the RING-type PEX proteins in plants. Additionally, testing the efficiency of PEX5 ubiquitination and matrix protein import in *pex12* Arabidopsis will help verify the mechanistic defects in protein import induced by reduced PEX12 levels.

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