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Elucidating matrix protein import and beta-oxidation pathways in plant peroxisomes

Bibi Rafeiza Khan
University of Missouri-St. Louis, brkwt9@umsl.edu

University of Missouri-St. Louis
Department of Biology

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by

Bibi Rafeiza Khan

M.S., Molecular Plant Pathology, University of Arkansas-Fayetteville, 2006
Dip. Ed., Education, University of Guyana, Guyana, 2004
B.S., Biology and Chemistry, University of Guyana, Guyana, 2002.

Advisory Committee

Bethany K. Zolman, Ph.D (Advisor)
Elizabeth A. Kellogg, Ph.D.
Lisa Schechter, Ph.D.
Sam Wang, Ph.D.

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Abstract

Plant peroxisomes function in fatty acid β-oxidation, jasmonic acid (JA) synthesis, and conversion of indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA). Peroxisomes lack genetic material; proteins required for peroxisomal processes are imported posttranslationally. The PEX5 and PEX7 receptors import proteins containing one of two peroxisomal targeting signals (PTS1 or PTS2). I took a genetic approach to better understand protein import and to elucidate the role of Acyl-CoA Oxidase (ACX) enzymes in the model plant *Arabidopsis thaliana*.

ACX enzymes catalyze the first step in β-oxidation. There are six *ACX* genes in Arabidopsis and each ACX enzyme acts on specific chain-length targets, but in a partially overlapping manner. To elucidate the substrate specificity of ACX enzymes and examine their roles *in planta*, I generated higher order *acx* mutants. *ACX* gene expression studies show distinct patterns between Col-0 and Ws and the enzymes showed altered activity during development. *acx* triple mutants showed reduced seed-storage mobilization and resistance to IBA. Several mutants specifically showed reduced fertility and the *acx1acx3acx5* defect was rescued with JA. Analysis of the higher order *acx* mutants has allowed me to determine which ACXs act in specific peroxisomal β-oxidation pathways.

To elucidate the mechanism of peroxisome matrix protein import, I studied an insertion mutant in the *PEX5* receptor. This mutant, designated *pex5-10*, makes a truncated pex5 protein, has germination defects and is dependent on sucrose for establishment. PTS1 and PTS2 protein import and enzymatic processes are also disrupted. To specifically

study PTS1 protein import, I created a pex5 mutant lacking the PTS1 binding region. This mutant rescued the PTS2 import defects in *pex5-10*. The *pex5-10* and PTS2 rescued lines allowed me to separate specific processes affected by import of PTS1 or PTS2 proteins.

This study has contributed to our understanding of the differences in the *ACX* genes and proteins in different Arabidopsis accessions and the role each protein plays in peroxisomal processes. The study also provides a better understanding of PTS1 and PTS2 proteins in specific peroxisomal pathways. Together these studies will contribute to elucidating the mechanism for the requirement for peroxisomal processes in plant growth and development.

Table of contents

Figures and Tables

CHAPTER 1: Introduction

1.1. Peroxisomes

1.1.a. Peroxisome identification and structure

Peroxisomes were first identified in 1965 as cellular organelles by Christian de Duve (De Duve, 1965). Isolation of compounds from liver cells and other tissues showed that peroxisomes were saturated with oxidative enzymes, peroxidase, catalase, glycolic acid oxidase and urate oxidase (De Duve, 1965). The name peroxisome was coined because this organelle is involved in inactivation of hydrogen peroxide. Peroxisomes are found in almost all eukaryotic cells except for members of the archezoa like the intestinal parasite *Giardia lamblia* (Cavalier-Smith, 1987). The morphology of peroxisomes differs between species, but they are typically between 0.1-1μm in diameter with a single lipid bilayer. The lipid bilayer membrane separates the peroxisome matrix from the cytoplasm (Fig.1-1) (Johnson and Olsen, 2001). There are 70-100 peroxisomes in a cell and they lack genetic material (Douglass SA, 1973; Kamiryo T, 1982). Plant peroxisomes are often placed into three groups: glyoxysomes, leaf peroxisomes and un-specialized peroxisomes. Glyoxysomes are the site for the glyoxylate cycle and are primarily found in seedlings. Leaf peroxisomes are found in leaves and are the site for β-oxidation and glycerate pathways of photorespiration.

1.1.b. Peroxisome biogenesis

Proteins required for peroxisome functions are imported posttranslationally into the peroxisome matrix. Genes that encode proteins for peroxisome biogenesis or function are referred to as *PEROXIN* (*PEX*) genes. More than 30 *PEX* genes have been isolated in yeast (Saraya et al., 2010). However, only a subset of *PEX* genes or homologs have been identified in plants: *PEX1* (Lopez-Huertas et al., 2000), *PEX2* (Hu et al., 2002), *PEX3* (Gerhardt, 1992), *PEX4* (Charlton and Lopez-Huertas, 2002), *PEX5* (Zolman et al., 2000), *PEX6* (Kaplan et al., 2001), *PEX7* (Schumann et al., 1999), *PEX10* (Schumann et al., 2003), *PEX11* (Lingard and Trelease, 2006), *PEX12* (Charlton and Lopez-Huertas, 2002), *PEX13* (Mano et al., 2006), *PEX14* (Lopez-Huertas et al., 1999), *PEX16* (Lin et al., 1999), *PEX19* (Charlton and Lopez-Huertas, 2002) and *PEX22* (Zolman et al., 2005).

The currently accepted model for peroxisome biogenesis suggests that peroxisomal proteins are synthesized on free polyribosomes in the cytosol. This model is referred to as the "growth and division" model for peroxisome biogenesis. In the growth and division model, proteins are imported posttranslationally into the peroxisome membrane or matrix and the size of the peroxisome increases (Lazarow and Fujiki, 1985; Purdue and Lazarow, 2001; Lazarow, 2003; Zhang and Hu, 2010). Mature peroxisomes then divide to generate more peroxisomes (Lazarow and Fujiki, 1985; Purdue and Lazarow, 2001; Lazarow, 2003; Zhang and Hu, 2010). The membrane of the peroxisome is formed by "buddings" from the rough endoplasmic reticulum (ER) (Fig. 1-2).

Proteins that function in the peroxisome membrane (PMP) are divided into different groups (Titorenko and Rachubinski, 2001). In the ER, group I PMPs are sorted and assembled prior to targeting to the peroxisome membrane (Fig.1- 2). Ascorbate peroxidase is a group 1 PMP and microscopy studies have shown that this protein is only observed in certain places in the ER (Mullen et al., 1999). This observation supports the hypothesis that after translation in the cytosol, group 1 proteins are targeted to the ER membrane for sorting. The term peroxisomal ER (pER) was suggested for this ER found in plants (Mullen et al., 2001). pER is part of the rough ER membrane (Lisenbee et al., 2003). Targeting of ascorbate peroxidase from the cytosol to the ER requires ATP and Hsp 70 (Mullen et al., 1999). Group 1 PMPs require two types of targeting signals to get to the peroxisome membrane. The first signal targets these proteins to the membrane of the ER while the second signal transports them from the ER to the peroxisome membrane (Baerends et al., 1996; Elgersma et al., 1998; Mullen and Trelease, 2000; Kim et al., 2006).

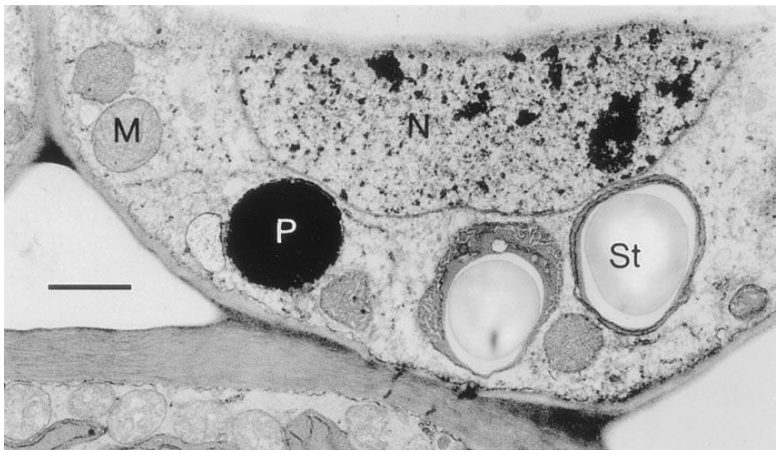


Figure 1-1. Electron micrograph showing the cytochemical localization of uricase in a peroxisome in mature cowpea nodules. M, Mitochondrion; N, nucleus; P, peroxisome; St, starch-containing plastid. (Taken from (Webb and Newcomb, 1987); (Johnson and Olsen, 2001).

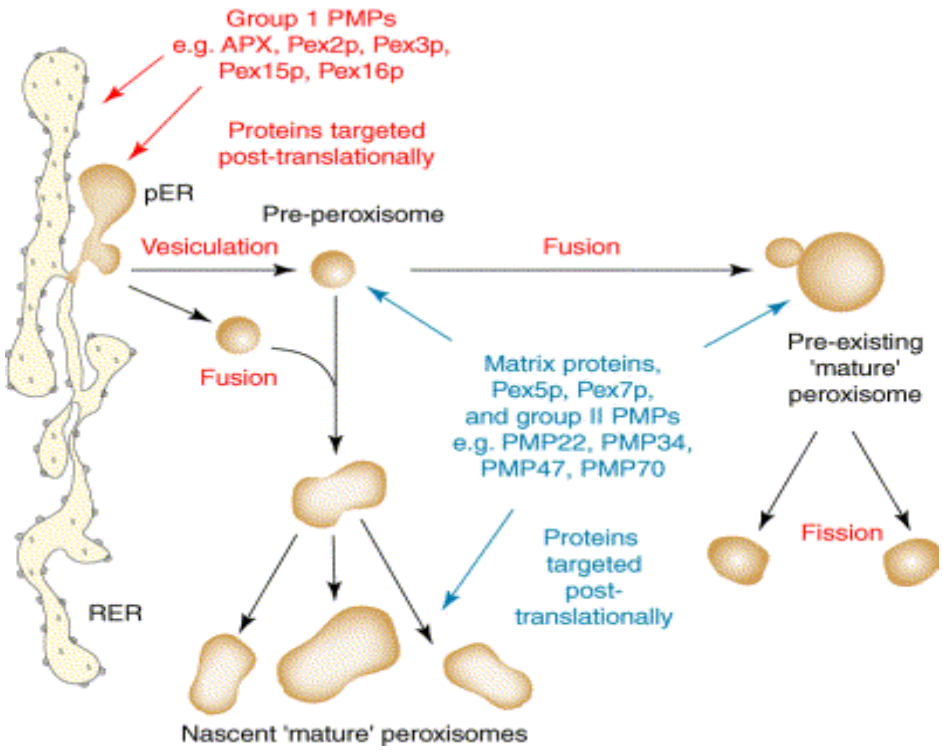


Figure 1-2. Model for peroxisome biogenesis. (Mullen et al., 2001).

1.1.c. Peroxisome matrix protein import

Because peroxisomes lack genetic material, proteins acting in peroxisomal processes are synthesized on free ribosomes in the cytosol and imported post-translationally (Olsen, 1998; Lazarow, 2003). These proteins contain one of two well-characterized peroxisomal targeting signals, PTS1 and PTS2, that target the protein to the peroxisomal matrix (Heiland and Erdmann, 2005; Platta et al., 2005; Hayashi M, 2006) . The PTS1 is a carboxyl-terminal tripeptide motif consisting of the consensus sequence (S/A/C)(K/R/H)(L/M) (Lametschwandtner G, 1998); in plants, the preferred targeting signal is serine-lysine-leucine (SKL) (Reumann et al., 2007). The SKL signal is retained after import, but sometimes can undergo specific processing like the sterol carrier protein x in hamsters (Kurochkin et al., 2007). The PTS2 is a more traditional signal found at the amino-terminus of the protein, consisting of nine amino acids with a consensus sequence of RL-X_5-HL (Lazarow, 2006). The most conserved pattern for plant PTS2 is RL-X_5 – HL (Reumann et al., 2004). The PTS2 is proteolytically cleaved after import. In the Arabidopsis genome, about 300 of the genes code for proteins with PTS1 or PTS2 signals (Kamada et al., 2003; Reumann, 2004; Reumann et al., 2007). A majority of these proteins contain the PTS1 sequence with about a quarter with the PTS2 sequence (Kamada et al., 2003; Reumann, 2004; Reumann et al., 2007)

PEX5 and PEX7 function as receptors that recognize PTS1 and PTS2 proteins, respectively, for matrix protein import (Fig.1-3) (Subramani, 1998; Baker, 2005; Hayashi M, 2006). PTS1 protein import occurs when the PTS1-containing protein is bound by PEX5 in the cytoplasm. The receptor-cargo complex interacts with the peroxisome

membrane through a docking complex of PEX13 and PEX14 (Fig.1-3) (Hayashi et al., 2000; Nito et al., 2002; Mano et al., 2006). The complex then translocates the matrix protein into the peroxisome, in a move that requires the RING-finger proteins PEX2, PEX10 and PEX12 (Fig.1-3) (Hu, 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005; Platta, 2009). The mechanism of exactly how this translocation event occurs *in planta* is still unknown. However, recent studies with PEX5 in tobacco cells demonstrated that membrane-associated PEX5 and one of the docking proteins, PEX14 form a gated ion channel for receptor-cargo translocation (Mast et al., 2010; Meinecke et al., 2010). The RING-finger proteins have also been implicated in recycling of the PEX5 receptor from the peroxisome matrix to the cytosol (Heiland and Erdmann, 2005). After translocation into the matrix, the cargo is released and receptors are recycled to the cytosol for additional rounds of import (Marzioch et al., 1994; Dodt and Gould, 1996; Dammai and Subramani, 2001; Nair et al., 2004; Brown, 2008).

PTS2 proteins are imported by the PTS2 receptor PEX7, which binds the PTS2 sequence in the cytosol (Fig.1-3) (Brown, 2008). However, PEX7 relies on PEX5 for import of its PTS2 protein cargo, perhaps in a piggy-backing type mechanism (Fig.1-3) (Nito et al., 2002; Hayashi et al., 2005; Woodward and Bartel, 2005). PEX7 also requires PEX14, PEX13 and PEX12 for import (Fig.1-3) (Singh, 2009). Recent evidence indicates that PEX5 also relies on PEX7 for import in Arabidopsis (Ramón NM, 2010), perhaps accelerating the import of both pathways.

PEX5 is a member of the tetratricopeptide repeat protein (TPR) family, a group of proteins with 34 amino acid repeats (Lamb, 1995). The TPR and a helical bundle are located at the C-terminal region of the PEX5 protein and together form a ring like structure upon ligand binding (Gatto et al., 2000; Stanley WA, 2006). The TPR region of the protein is responsible for interaction with the PTS1 protein for import. PEX5 also contains ten pentapeptide repeats (PPR) and this domain docks the receptor to the peroxisome membrane for import. There is also a known PEX7 binding domain in the PEX5 protein for binding of PEX7 with its PTS2 cargo proteins.

1.1.d. Monoubiquitination may target PEX5 for recycling

In yeast, monoubiquitination of PEX5 by PEX12 in the peroxisome matrix targets the receptor for recycling to the cytosol and polyubiquitination by PEX2 results in degradation (Platta, 2009). Recycling of the protein requires the PEX4 E2 protein (anchored to the peroxisome membrane by PEX22), and the PEX1 and PEX6 ATPases (Faber et al., 1998; Geisbrecht et al., 1998; Kiel et al., 1999; Zolman and Bartel, 2004; Zolman et al., 2005) (Fig. 1-4). PEX1 and PEX6 interact and this interaction requires ATP. In *S. cerevisiae* and mammals, PEX6 is docked to the peroxisome membrane by PEX15 and PEX26, respectively; however, in plants the proteins for docking of PEX6 have not been identified. PEX6, PEX4 and PEX22 were also hypothesized to be involved in PEX5 recycling in *Arabidopsis* (Zolman and Bartel, 2004; Zolman et al., 2005). While PEX5 import is ATP independent, recycling is dependent on ATP hydrolysis, which is facilitated by PEX1 and PEX6.

In eukaryotic organisms, addition of an ubiquitin moiety to a protein is a process that requires three enzymes: ubiquitin activating, conjugating and ligase enzymes (Özkan et al., 2005; Michelle et al., 2009). The result of ubiquitination is dependent on whether the protein is polyubiquitinated (the conjugation of four or more ubiquitin moieties) or monoubiquitinated. Polyubiquitination targets the protein for degradation by the 26S proteasome (Thrower et al., 2000), while monoubiquitinated proteins are not targeted for degradation. Monoubiquitinated proteins result in a change in activity or cellular localization (Hicke, 2001). Previous studies in yeast showed that PEX5 can be ubiquitinated (Kiel et al., 2005; Kragt et al., 2005). The Ubiquitin-conjugating enzymes, UBC4 and UBC5, located at the peroxisome membrane polyubiquitinate PEX5 (Platta et al., 2004; Kiel et al., 2005). In *pex4*, *pex22*, *pex1*, *pex6* and *pex15* deletion mutants, polyubiquitination of yeast PEX5 requires UBC4-UBC5 (Platta et al., 2004; Kragt et al., 2005). Thus, in yeast PEX1, PEX4, PEX6, PEX15 and PEX22 are involved in PEX5 import late in the cycle. These proteins are important for PEX5 recycling to the cytoplasm (Collins et al., 2000; Platta et al., 2005). Thus, when PEX5 recycling is blocked, polyubiquitination of PEX5 in *pex4*, *pex22*, *pex1*, *pex6* and *pex15* may occur to remove PEX5 from the peroxisome membrane (Platta et al., 2004; Kiel et al., 2005). Mono-ubiquitination of PEX5 requires PEX2 and PEX12 (Platta et al., 2009). Thus, it was hypothesized that PEX22 docks the ubiquitin conjugating enzyme, PEX4, to the peroxisome membrane where it interacts with the PEX2, PEX10 and PEX12, which might function as ubiquitin ligases resulting in ubiquitination of PEX5 (Platta et al., 2009). Thus, since PEX5 can be either mono- or polyubiquitinated, it was suggested that

polyubiquitination targets PEX5 for degradation while monoubiquitination is the signal that facilitates PEX5 recycling.

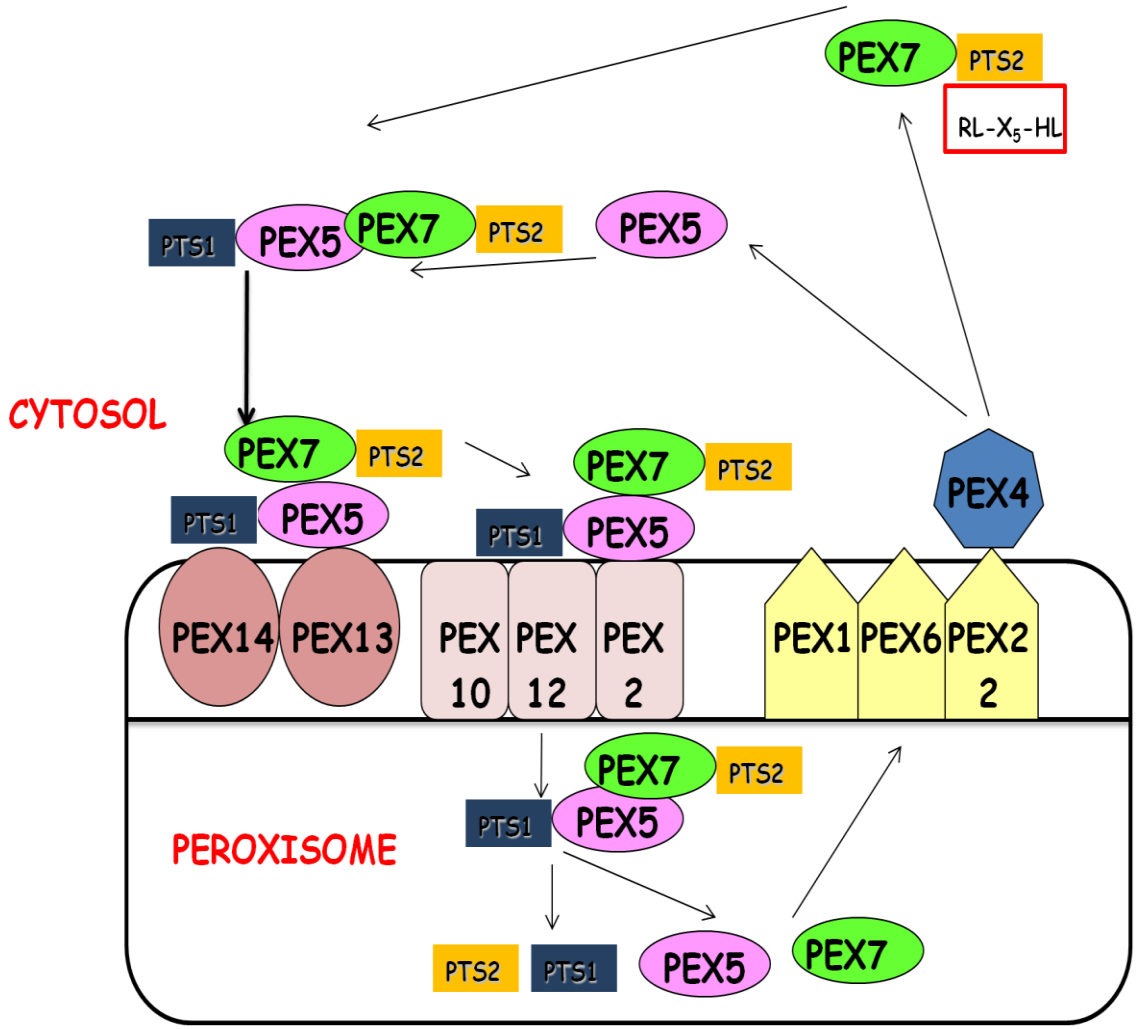


Figure 1-3. Model of PTS1 and PTS2 protein import into the peroxisome matrix.

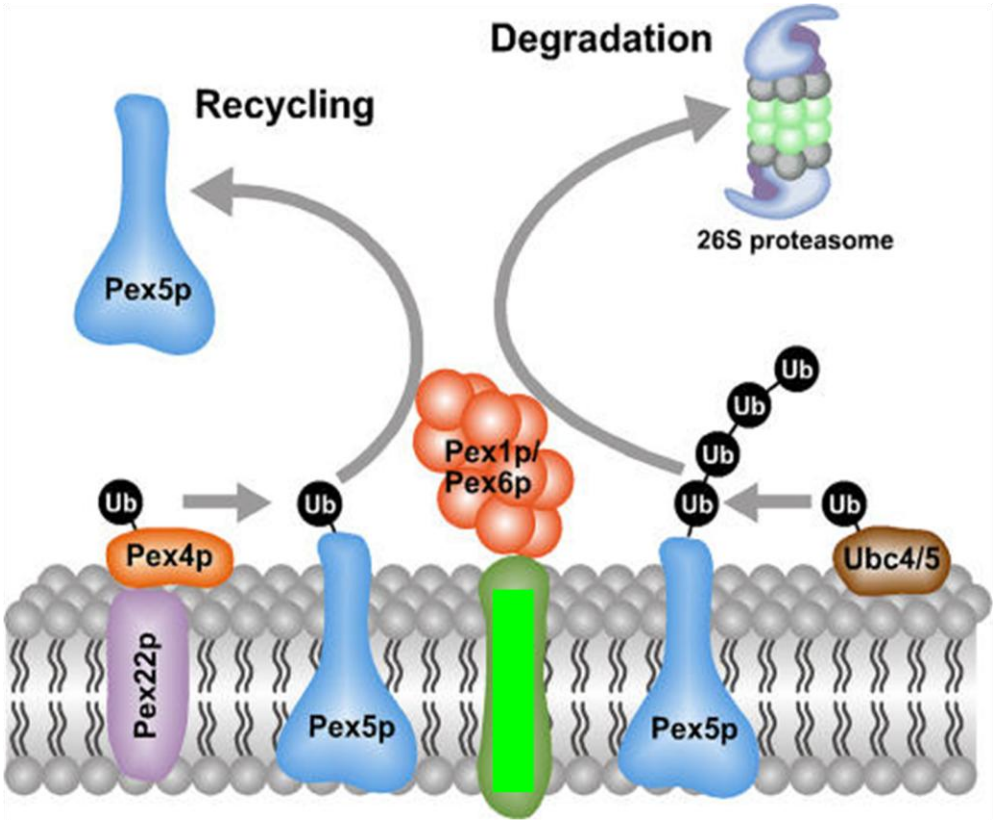


Figure 1-4. Model of PEX5 recycling. (Platta et al., 2007)

1.1.e. Plant peroxisomal function

In eukaryotic organisms, peroxisomes function in the compartmentalization of a large number of metabolic processes. Perhaps, the most notable function of peroxisomes is the detoxification of hydrogen peroxide (Cooper, 1971). Peroxisomes also play a major role in plant hormone β-oxidation and they are the major site for fatty acid β-oxidation. They are the site for the conversion of an endogenous storage form of auxin, indole-3-butyric acid (IBA), to its active signaling counterpart indole-3-acetic acid (IAA) (Zolman et al., 2000; Adham et al., 2005). The latter part of the jasmonic acid (JA) synthesis pathway takes place in peroxisomes (Wasternack and Kombrink, 2010). Metabolism of intermediates such as propionate, isobutyrate, acetate and benzoic acid occurs in peroxisomes (Zolman et al., 2001; Hooks et al., 2007; Lucas et al., 2007; Ibdah and Pichersky, 2009). Peroxisomes are also important in physiological processes such as senescence, starvation, photorespiration, photomorphogenesis and stress tolerance (Wanner et al., 1991; Graham IA, 1992; Hu et al., 2002; Foyer, 2009; Kunza, 2009) (Donga et al., 2009).

1.2. PLANT PEROXISOMAL PROCESSES

1.2.a. Peroxisomal fatty acid β-oxidation

1.2.a.i. Fatty acid storage and mobilization

During seed development, nutrients are stored for use during germination and early seedling establishment prior to photosynthesis. Seeds are an important source of food for both humans and animals (Bewley and Black, 1994). It is estimated that approximately

70% of food comes from seeds (Bewley and Black, 1994). During seed development, carbon, in the form of triacylglycerol (TAG), is stored for later use. In Arabidopsis seeds, starch is converted to TAG and stored in liposomes. About 45% of the dry weight of a mature Arabidopsis seed is made up by liposomes (O'Neill et al., 2003). The TGAs are metabolized by lipase in the cytosol to produce various length fatty acids (Pinfield-Wells et al., 2005). This process occurs during germination and the acetyl-CoA is converted to citrate and then sugars, to be used either for respiration or gluconeogenesis for growth and development (Fig. 1-5).

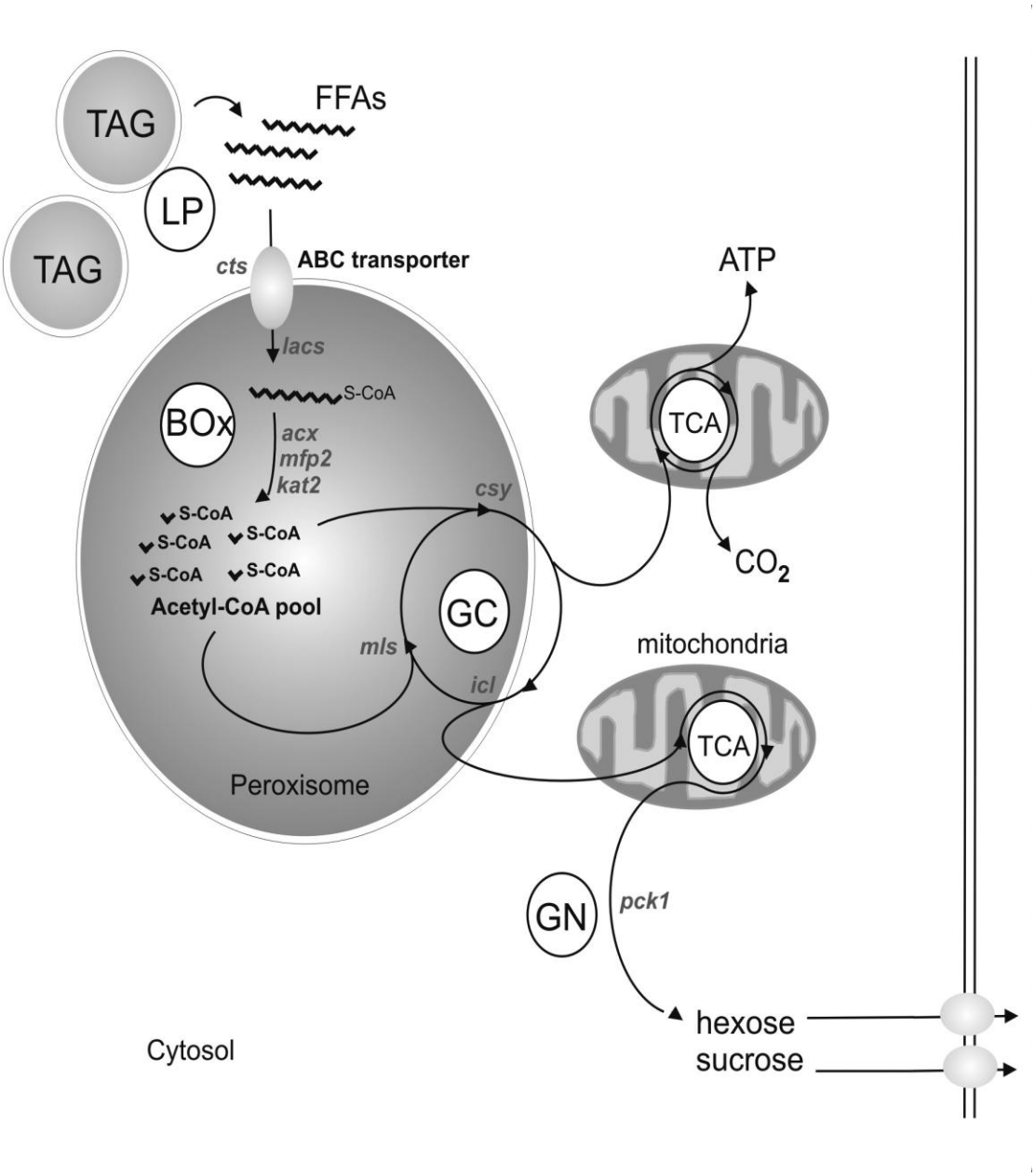


Figure 1-5. Major metabolic pathway for lipid mobilization in germinating Arabidopsis seed. (Pinfield et al., 2006).

1.2.a.ii. Fatty acid transport into the peroxisome

Fatty acids derived from TAG are transported into the peroxisome for ß-oxidation. The transporter, CTS, PXA1 or PED3 is used to transport fatty acid into the peroxisome (Zolman, 2001a; Footitt, 2002; Hayashi et al., 2002). Mutations in the *PXA1* gene resulted in mutants that were compromised in fatty acid β-oxidation. This observation suggest that the PXA1 protein is important for transport of fatty acids into the peroxisome (Zolman, 2001a; Footitt, 2002; Hayashi et al., 2002). *pxa1* mutants also accumulated long chain acyl-CoA esters, indicating a disruption in either acyl-CoA transport or metabolism (Footitt, 2002). These mutants also showed significantly impaired germination defects suggesting that this gene also regulates dormancy and germination (Kanai et al., 2010).

1.2.a.iii Fatty acid activation

Prior to metabolism, fatty acids are conjugated to CoAs by the long chain acyl-CoA synthetases enzyme (LACS) (Shockey et al., 2002) (Fig. 1-5). This reaction occurs in two steps and the first step results in the formation of adenylate acyl-AMP. The carbonyl carbon of the adenylate then reacts with the thiol group of CoA resulting in acyl-CoA and releasing AMP (Groot et al., 1976). There are two LACS enzymes in Arabidopsis in the peroxisome (Fulda et al., 2002). In 2004, the role of LACS6 and LACS7 in fatty acid metabolism was shown (Fulda et al., 2004). The *lacs6 lacs7* double mutants were defective in seedling establishment and fatty acid β-oxidation, indicating that fatty acid activation occurs inside the peroxisome. Therefore, the substrate of PXA1 is free fatty acids, rather than acyl-CoA esters (Fulda et al., 2004). Alternatively, acyl-CoA esters

are transported into the peroxisome and cleaved to release free fatty acids, which are then primed by LACS for fatty acid for β-oxidation (Fulda et al., 2004).

1.2.a.iv. The first step in fatty acid β-oxidation is catalyzed by Acyl-CoA oxidase

After fatty acids are primed by LACS for β-oxidation by the addition of CoAs, acyl-CoA oxidases (ACXs) then catalyze the first step of the ß-oxidation pathway. In this reaction, the acyl CoA is β-oxidized to 2-trans-enoyl-CoA (Fig 1-6). FAD acts as a co-factor in this reaction and $FADH_2$ is generated as a byproduct. The $FADH_2$ is then broken down by the enzyme flavoprotein dehydrogenase to produce hydrogen peroxide.

Six *ACX* genes were identified in Arabidopsis. ACX enzymes have distinct but slightly overlapping fatty acid chain length specificities (Hooks et al., 1999). The substrate specificity for four of the ACX proteins was biochemically determined. ACX1 enzyme catalyzes oxidation of long-chain fatty acids (8-20 carbons), ACX2 oxidizes fatty acids with very-long chain (14-20 carbons), ACX3 has a preference for fatty acids with medium-chain length (8-14 carbons) and ACX4 catalyzes the oxidation of short-chain (4-6 carbons) fatty acids (Hayashi et al., 1999; Hooks et al., 1999; De Bellis et al., 2000; Eastmond et al., 2000; Froman et al., 2000). *ACX3* and *ACX6* share a sequence similarity of 87% while ACX1 and ACX5 share a 85% similarity. Thus, the ACX enzymes may exhibit functional redundancy. *ACX1*, *ACX2*, *ACX3*, *ACX4* and *ACX5* are all expressed in seedlings but *ACX2* and *ACX3* have the highest level of expression in seedlings and decline as the plant develops. *ACX6* transcripts have not been detected in plants and no

phenotypes have been observed in plants mutated for *ACX6* (Yamada et al., 2003) (Adham et al., 2005). This suggests that *ACX6* is not expressed in Arabidopsis.

None of the *acx* single mutants except *acx4* displayed a sucrose dependent phenotype, indicating that peroxisomal fatty acid β-oxidation is not compromised in these mutants (Adham et al., 2005). *acx4* was sucrose dependent in the light (Adham et al., 2005). However, *acx1acx2* double mutants display a sucrose-dependent phenotype (Adham et al., 2005). Arabidopsis stores primarily very long chain fatty acids for energy during germination and ACX2 has a preference for very long chain fatty acids. However, ACX1 can also metabolize long chain fatty acids, so the sucrose dependence phenotype of *acx1acx2* double mutant is due to the overlapping substrate preference of ACX1 and ACX2.

Mutations in *acx2, acx5* and *acx6* did not result in a phenotype (Adham et al., 2005). However, double mutant combinations of *acx1acx2* and *acx1acx5* result in enhanced root elongation on inhibitory concentrations of IBA, suggesting that ACX2 and ACX5 in combination with ACX1, ACX3 and ACX4 affect IBA (Adham et al., 2005). Previous studies showed that an *acx3acx4* double mutant in the Ws ecotype is embryo lethal (Rylott et al., 2003). This finding indicates that peroxisomal β-oxidation is critical for embryo development (Rylott et al., 2003). However, since ACX enzymes also act in IBA and jasmonic acid β-oxidation pathways, exactly which β-oxidation pathway is essential for embryogenesis is yet to be determined.

Thus, since *acx1*, *acx3* and *acx4* demonstrate resistance to root elongation inhibition of IBA and *acx1acx3* double mutants inhibit lateral root formation, it is reasonable to argue that ACX1, ACX3 and ACX4 are the three most active ACX enzymes affecting IBA β-oxidation and they may be involved in IBA metabolism. On the other hand, since *acx1acx2*, *acx1acx3* and *acx1acx5* double mutants display enhanced resistance to the inhibitory effects of IBA on root elongation, it is possible that ACX1, ACX2, ACX3, ACX4 and ACX5 are indirectly blocking IBA metabolism (Adham et al., 2005). One proposed indirect model for this process is that when ACX enzyme activity is reduced, fatty acyl-CoAs are no longer effectively oxidized, thus the CoA pool present in the peroxisome is sequestered by fatty acids, thereby decreasing the CoA available to activate IBA to its active form IBA-CoA (Adham et al., 2005).

1.2.a.v. Multifunctional protein in fatty acid β-oxidation

The Multifunctional protein (MFP) catalyzes the second and third steps in fatty acid β-oxidation (Fig 1-6). There are two MFP isoforms in Arabidopsis: ABNORMAL INFLORESCENCE MERISTEM1 (AIM1) and MFP2 (Richmond and Bleecker, 1999). MFP possess four different activities: 2-trans enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, hydroxyacyl-CoA epimerase, and 3,2-enoyl-CoA isomerase (Behrends et al., 1988; Giihnemann-Schiifer and Kindl, 1995). MFP use different combinations of activities for the ß-oxidation of saturated and unsaturated fatty acids. MFP have 2-trans enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities on saturated fatty acids. *aim1* mutants showed no seedling phenotype, but adult plants showed stunted vegetative growth with abnormal leaves and inflorescences

(Richmond and Bleecker, 1999). The most dramatic phenotype of *aim1* is infertility. *mfp2* mutants were sucrose dependent with short hypocotyls in the dark (Rylott et al., 2006). *aim1 mfp2* double mutants were embryo lethal (Rylott et al., 2006). Despite the differences in phenotypes both MFP2 and AIM1 can efficiently break down long chain enoyl-CoA substrates (Arent et al., 2010).

1.2.a.vi. Final step in fatty acid β-oxidation

The final step of fatty acid ß-oxidation is catalyzed by the enzyme, 3-ketoacyl-CoA thiolase (PED1/ KAT2) (Fig 1-6) (Hayashi et al., 1998). In this reaction, 3-ketoacyl-CoA undergoes a thiolytic cleavage to acyl-CoA and acetyl-CoA. Three thiolase genes with putative peroxisomal targeting signals were identified in Arabidopsis (Germain et al., 2001). *ped1*was isolated in a screen for auxin synthesis mutants (Hayashi et al., 1998). PED1/KAT2 is the only thiolase expressed during germination and early seedling development (Germain et al., 2001). The *ped1/kat2* mutant was sucrose dependent with defects in seedling establishment and resistance to the IBA analog 2, 4-dichlorophenoxybutyric acid (2,4DB). Recent in vitro studies demonstrated interaction between KAT2 and MFP (Pye et al., 2010).

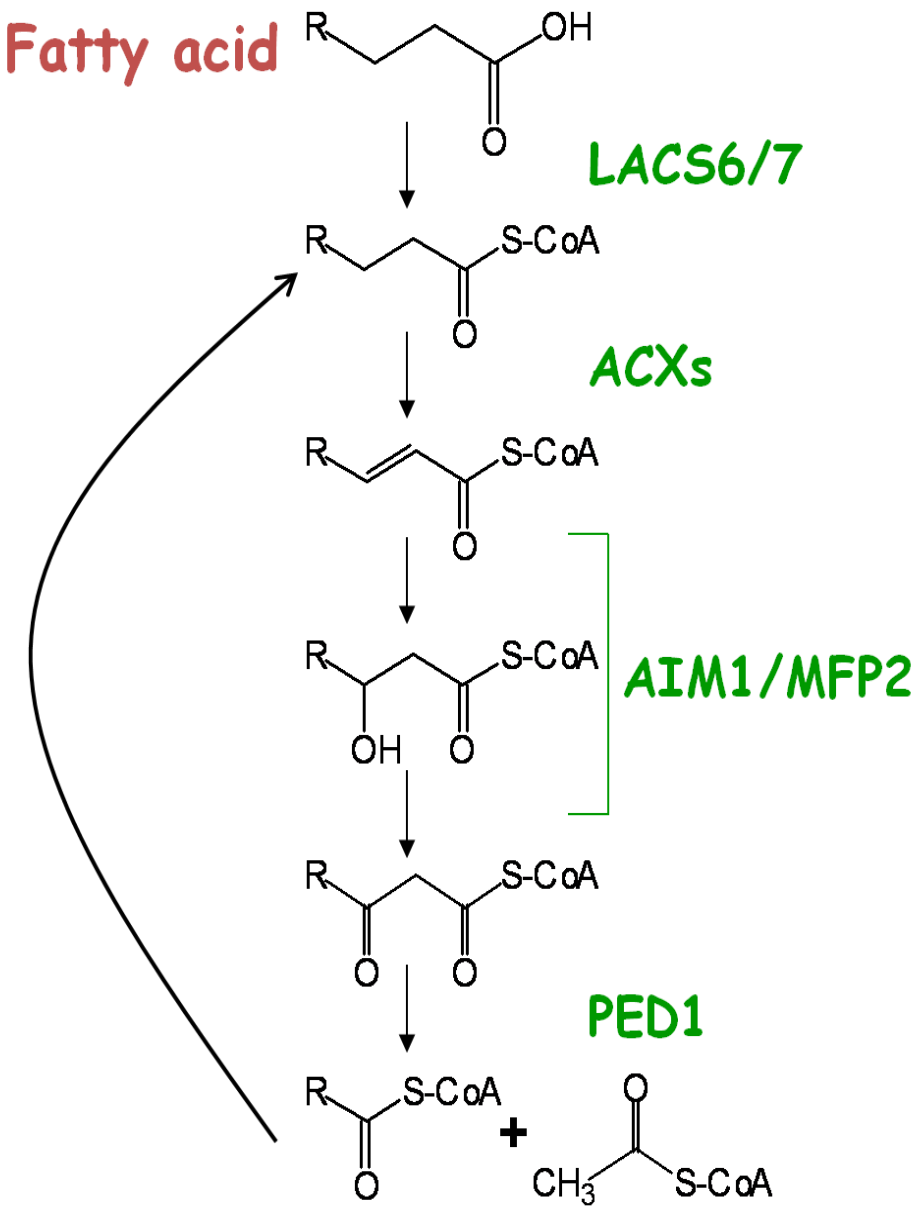


Figure 1-6. Pathway for fatty acid β-oxidation.

1.2.b. Auxins

1.2.b. i. Auxin identification and isolation

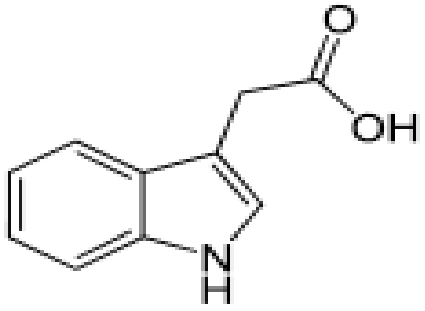
Indoleacetic acid (IAA) was first discovered in 1885 (Salkowski, 1985) and since its discovery it has been studied extensively. In 1926, IAA was isolated in coleoptile tips taken from Avena stems (Went, 1926).

1.2.b.ii. Endogenous and synthetic auxins

Compounds that stimulate cell elongation and have other physiological activities similar to IAA are classified as auxins (Mauseth, 1991; Raven, 1992). There are many forms of auxins, but the most abundant endogenous auxin is IAA. Additional naturally occurring auxins include 4-chloroindole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA) and indole-3-butyric acid (IBA) (Fig1-7). Some synthetic auxins are 1-naphthaleneacetic acid (NAA), 2, 4- dichlorophenoxyacetic acid (2,4-D), 2-Methoxy-3,6-dichlorobenzoic acid (dicamba) and 4-Amino-3,5,6-trichloropicolinic acid (tordon or picloram) (Fig1-8).

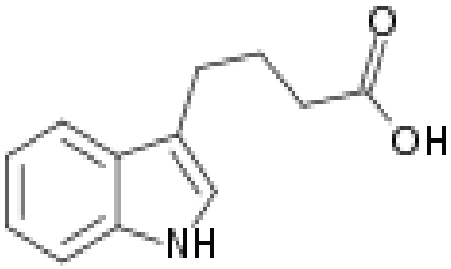
1.2.b.iii. Role of auxins in plants

Many aspects of plant growth and development are affected by auxins. Some functions of auxins in plants are stimulation of cell elongation, cell division, phloem and xylem differentiation, root initiation and lateral root development. Additional roles include suppression of lateral buds, delayed leaf senescence, inhibition or stimulation of leaf and fruit abscission, delayed fruit ripening and promotion of female flower development (Mauseth, 1991; Raven, 1992; Davies, 1995).



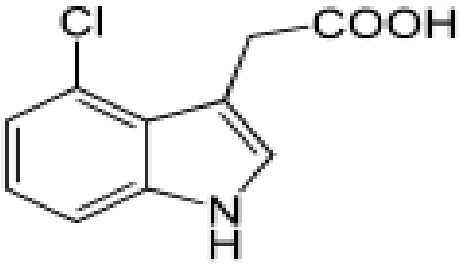
Indole-3-acetic acid

IAA



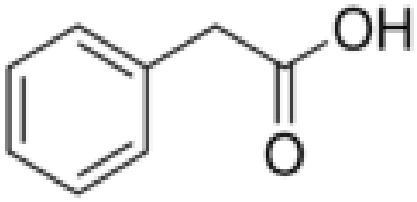
Indole-3-butyric acid

IBA



4-chloroindole-3-acetic acid

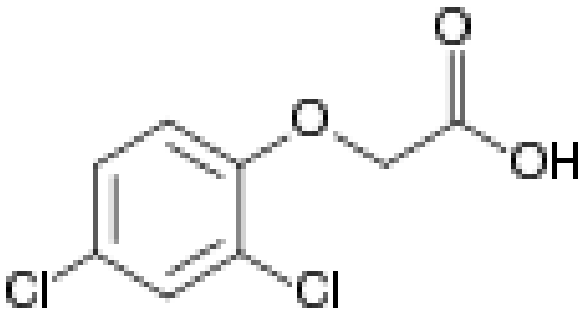
(4-CI-IAA)



2-phenylacetic acid

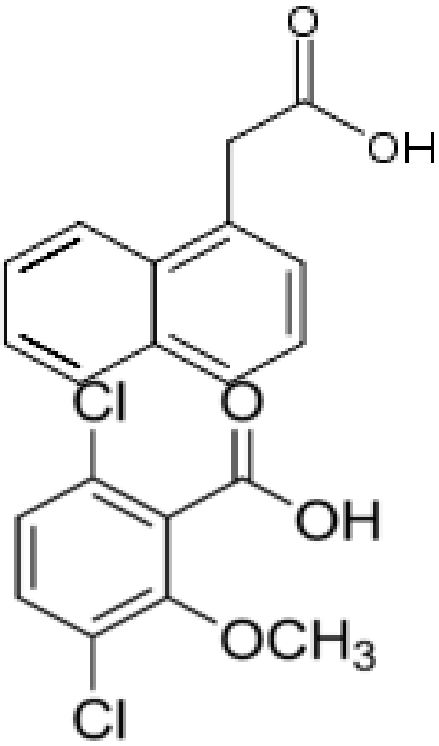
(PAA)

Figure 1-7. Structures of naturally occurring auxins



2,4-Dichlorophenoxyacetic acid

(2,4-D)

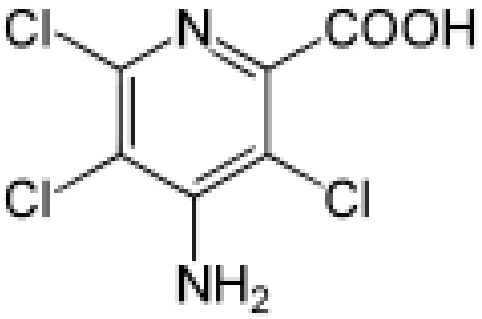


α-Naphthalene acetic acid

(α-NAA)

2-Methoxy-3,6-dichlorobenzoic acid

(dicamba)



4-Amino-3,5,6-trichloropicolinic acid

(tordon or piclorom)

Figure 1-8. Structures of synthetic auxins

1.2.b.iv. IAA biosynthesis

Plants can regulate the amount of IAA present in tissues either by regulating its synthesis, by forming conjugates, or by degradation. When IAA is conjugated to amino acids, they become inactive. When IAA is required in a particular tissue, the IAA conjugates are hydrolyzed to IAA, the active signaling hormone.

IAA is derived from the amino acid tryptophan via two mechanisms. In the first mechanism, tryptophan is transaminated to indolepyruvic acid. Indolepyruvic acid then undergoes decarboxylation to form indoleacetaldehyde. In the final step, indoleacetaldehyde is oxidized to IAA. In the second mechanism, tryptophan is decarboxylated to tryptamine then oxidized and deaminated to indoleacetaldehyde. Indoleacetaldehyde undergoes further oxidation to produce IAA.

1.2.b.v. IBA is converted to IAA in the peroxisome

Mutants defective in the IBA to IAA conversion pathway exhibit a phenotype of long roots on inhibitory concentrations of IBA, while remaining sensitive to IAA. Mutants defective in IBA metabolism were found by screening mutagenized seeds for long roots on inhibitory concentrations of IBA (Zolman et al., 2000). The enzymes that act in IBA metabolism are yet to be identified. Studies have shown that IBA, which is slightly less abundant than IAA in plants, is more effective in forming lateral roots (de Klerk et al., 1999). About 25% to 30% of the auxins present in Arabidopsis seedlings are IBA (Ludwig-Müller and Epstein, 1993). Root elongation is only partially inhibited at the concentration of IBA that initiate lateral root formation (Zolman et al., 2000). Root elongation is severely inhibited at the concentration at which IAA initiates lateral roots

(Zolman et al., 2000). Additionally, IBA is more stable than IAA in different light and temperature conditions (Nordström et al., 1991). Much is yet to be determined in terms of the molecular mechanism by which IBA functions. One school of thought suggests that IBA is converted to IAA in the peroxisome by a mechanism that is similar to peroxisomal β-oxidation of fatty acids (Fawcett et al., 1960; Zolman et al., 2000). In an Arabidopsis genetic screen, several IBA resistant and sucrose dependent mutants were isolated (Zolman et al., 2000). The dependence of these mutants on sucrose for seedling establishment suggests that the fatty acid β-oxidation is compromised, while the IBA resistant phenotype of these mutants supported the hypothesis that IBA is β-oxidized to IAA.

The first evidence that IBA may be β-oxidized to IAA in the peroxisomes was shown in 1960 (Fawcett et al., 1960). This study showed that indolealkenecarboxylic acid is metabolized either to an even or odd carbon chain length compound, acetate or propionic acid respectively (Fawcett et al., 1960). Since IBA has two carbons more than IAA, this result suggests that IBA is converted to IAA by a single round of β-oxidation. Additional studies showed that when different plants, such as bean (Wiesman et al., 1988), apple (van der Krieken et al., 1992), grape (Epstein and Lavee, 1984), maize (Ludwig-Müller and Epstein, 1991), olive (Epstein and Lavee, 1984), pea (Nordström et al., 1991) and pear (Baraldi et al., 1993) were treated with radioactively labeled IB- radioactively labeled IAA was obtained. IBA also forms conjugates with amino acids (Ludwig-Müller and Epstein, 1993). IBA and IAA can be inter-converted (Strader et al., 2010) (Fig 1-9), which suggests that IBA may act as a precursor to IAA.

Additional studies suggest that IBA may act directly as an auxin without being converted to IAA. One argument for this hypothesis is the observation that IBA is more effective than IAA, 2,4-D, or other forms of synthetic auxins in promoting lateral root formation (Ludwig-Müller, 2000). Further studies showed that endogenous IBA levels increase during lateral root formation on IBA media (Nordström et al., 1991; van der Krieken et al., 1992).

In the proposed mechanism for the conversion of IBA to IAA, two methylene groups are removed from IBA by a single round of β-oxidation thereby generating IAA (Zolman et al., 2000) (Fig1-10). Studies suggest that the reactions involved in β-oxidation of fatty acids are also involved in IBA oxidation (Zolman et al., 2000). These reactions include thioesterification, oxidation, hydration, dehydrogenation and thiolysis. The end result of IBA β-oxidation is IBA-CoA, which is then hydrolyzed to generate free IAA (Zolman et al., 2000). If IBA β-oxidation occurs similarly to fatty acid β-oxidation, then it is reasonable to hypothesize assume that the same enzymes that catalyze peroxisome β-oxidation of fatty acids may also catalyze IBA oxidation. Alternatively, a unique set of peroxisomal enzymes may catalyze the conversion of IBA to IAA.

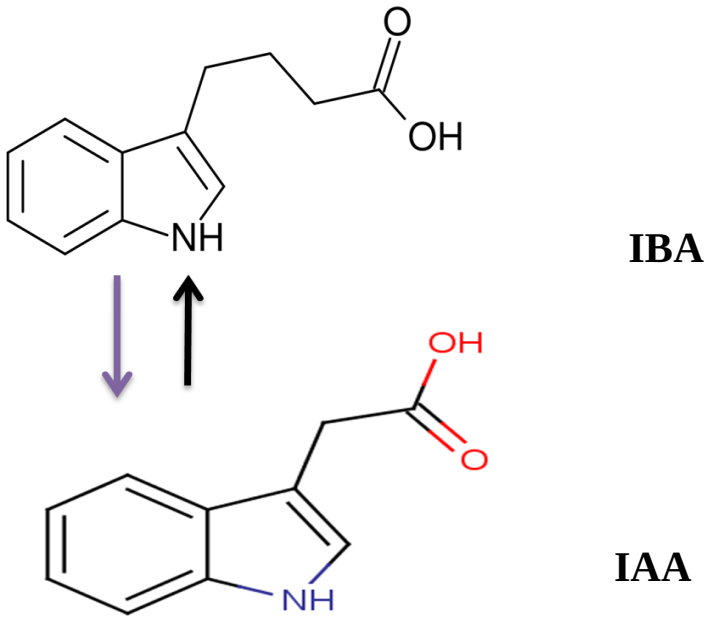


Figure 1-9. Interconversion of IAA to IBA

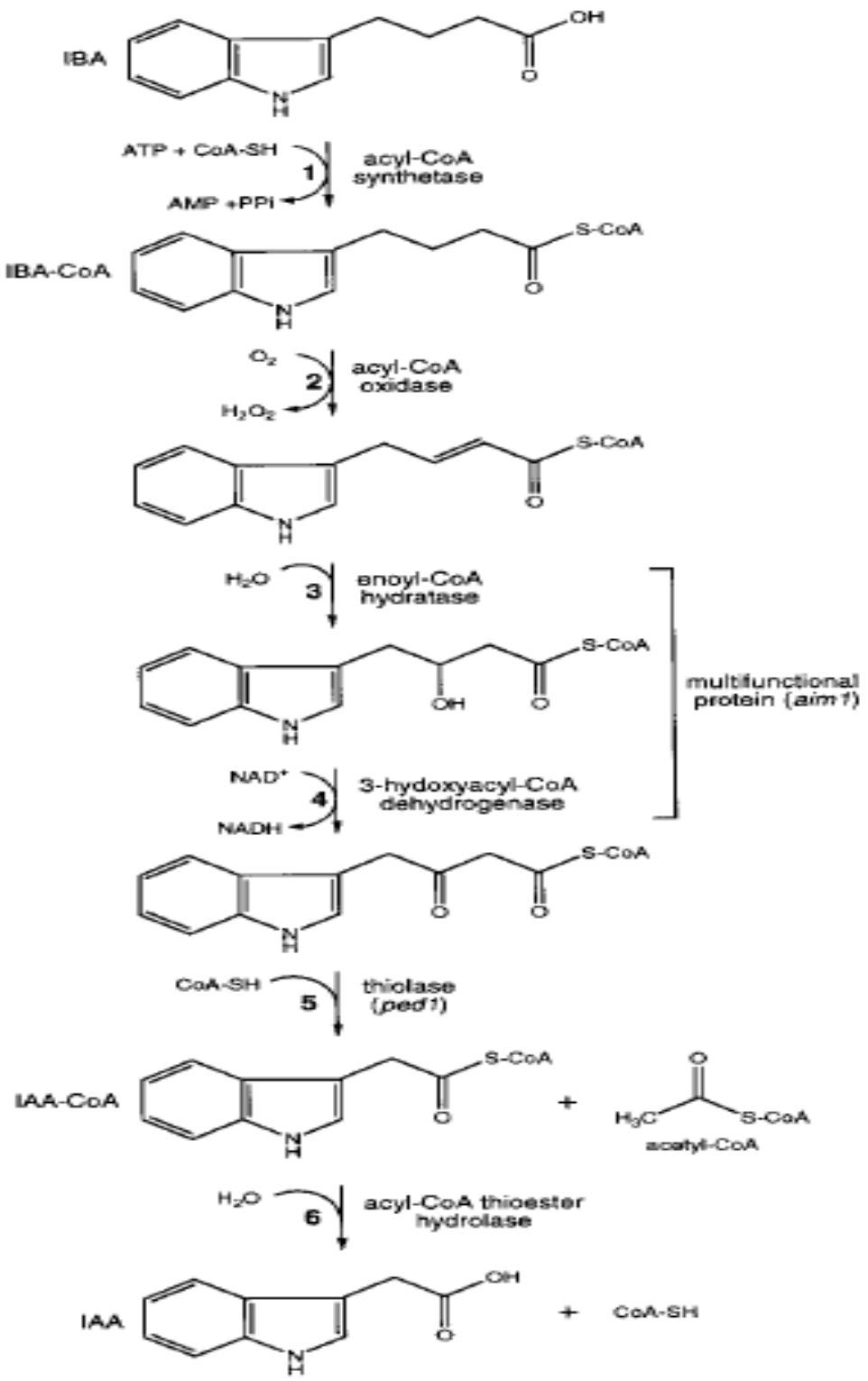


Figure 1-10. Proposed mechanism for conversion of IBA to IAA (Zolman et al., 2000).

1.2.b. vi. IBA transport

Several studies demonstrated slow polar movement of IBA in stems (Went and White, 1938; Leopold and Lam, 1961; Yang and Davies, 1999). These studies showed that when IBA is applied to the top of a shoot, several nodes developed around the base indicating basipetal IBA transport (Yang and Davies, 1999). Additional studies showed basipetal IBA transport by application of radiolabeled IBA to the base of the explants (Epstein and Lavee, 1984; Wiesman et al., 1988; Epstein and Sagee, 1992; van der Krieken et al., 1992; Epstein and Ackerman, 1993). Comparison of IBA and IAA transport in Arabidopsis inflorescences showed predominantly basipedal transport of these two auxins (Ludwig-Müller et al., 1995). IBA is transported basipetally in Arabidopsis hypocotyls and acropetally in root (Rashotte et al., 2003). These results showed that, at least in Arabidopsis, IBA transport occurs similar to IAA (Rashotte et al., 2000; Rashotte et al., 2001). However, in Arabidopsis inflorescences, no IBA transport was observed (Rashotte et al., 2003) in contrast to the basipetal transport of IAA in this tissue (Okada et al., 1991; Bennett et al., 1995). Examination of radiolabeled IBA and IAA movement in the presence of unlabeled IBA and IAA showed that both auxins were taken up at high concentration, suggesting that they are transported by proteins (Rashotte et al., 2003). Together these results suggest that IBA is transported by different proteins than IAA and that these proteins may have different tissue specificity (Rashotte et al., 2003). IBA tissue specific transport in Arabidopsis provides evidence that endogenous IBA is involved in growth and development in specific tissues. For example, IBA promotes lateral root formation (Poupart and Waddell, 2000; Zolman et al., 2000) and stem elongation (Yang and Davies, 1999).

1.2.b. vii. ACXs in IBA β-oxidation

Previous studies showed that root elongation of *acx3* and *acx4* mutants is not inhibited by 2,4-DB (Rashotte et al., 2003). 2, 4-DB is metabolized to the IAA analog 2, 4-dichlorophenoxyacetic acid (2,4-D) (Wain and Wightman, 1954). This result therefore suggests that fatty acid and 2, 4-DB metabolisms are similar, and perhaps the same enzymes act in both of these pathways. Further studies revealed that *acx1*, *acx3*, and *acx4* mutants are resistant to root elongation inhibition by IBA, while remaining sensitive to IAA (Adham et al., 2005). However, these *acx* single mutants initiate lateral roots similarly to Wt in the presence of IBA (Adham et al., 2005). *ACX3-GUS* expression in roots showed that *ACX3* expression is only slightly detectable in early lateral root primordia but expression increases after lateral roots emerge (Adham et al., 2005). This observation may explain why *acx3* mutants produce a normal numbers of lateral roots. Although the expression of the other *ACX* genes was not examined, it is possible that their expression pattern in roots is the same as *ACX3*. This would explain why *ACXs* can also form lateral roots similar to Wt. However, double mutants of *acx1* and *acx3* produce less lateral roots compared to Wt and individual single mutants (Adham et al., 2005). The *acx1acx3* double mutant also exhibits enhanced resistance to IBA root elongation inhibition (Adham et al., 2005).

1.2.c. Jasmonates

1.2.c.i. Identification of jasmonates

Jasmonates are a group of phytohormones derived from membrane fatty acid metabolism (Wasternack and Hause, 2002; Chen et al., 2006; Browse, 2009). Jasmonates, like jasmonic acid (JA) and methyl jasmonate (MeJA), play a role in plant defense responses and development (Seo et al., 2001; Arimura et al., 2007; Wasternack, 2007; Wasternack and Kombrink, 2010). JA can be modified in various ways by forming conjugates with different amino acids or by undergoing hydroxylation, forming compounds for the activation of jasmonate-responsive defense genes (Creelman and Mullet, 1997; Liechti and Farmer, 2006; Thines et al., 2007). JA plays a critical role in male floral development in Arabidopsis (Sanders et al., 2000; von Malek et al., 2002; Avanci et al., 2010) and tomato plants (Avanci et al., 2010).

1.2.c.ii. Jasmonate biosynthesis

The JA biosynthesis pathway was first described in 1984 (Vick, 1984). Since then, jasmonate biosynthesis and signaling pathways have been extensively studied in Arabidopsis and tomato and some monocots (Kazan and Manners, 2008).

JA and MeJA are produced from fatty acids α-linolenic acid (α-LeA, 18:3) and hexadecatrienoic acid (16:3) in the chloroplast membranes (Fig.1-11). Linolenic and hexadecatrienoic acids are stored as monogalactosyldiacylglycerol (MGDG) (Fig.1-11), which can then form lipids in chloroplast membranes through the activity of phospholipase A. The activity of phospholipase A is stimulated by wounding or

pathogen attacks. Linolenic and hexadecatrienoic acids are released in the chloroplast matrix and one molecule of oxygen is added by 13-lipoxygenase (LOX), forming 13-hydroperoxy-octadecatrienoic acid (13-HPOT) and 11-hydroperoxy-hexadecatrienoic (11-HPHT) (Fig.1-9). 13-HPOT and 11-HPHT are oxidized by allene oxide synthase (AOS), forming allene oxide, which is quickly converted to 12-OPDA and dnOPDA by the enzyme allene oxide cyclase (Fig.1-11).

12-OPDA and dnOPDA are precursors to JA and are the last products of the metabolic pathway of JA biosynthesis that takes place in the chloroplast. For the final steps in JA production, the 12-OPDA and dnOPDA molecules are transferred to the peroxisomes via the ABC COMATOSE (CTS) receptor (Rosahl and Feussner, 2005; Liechti and Farmer, 2006; Delker. C. et al., 2007; Browse, 2009)(Fig. 1-11). In the peroxisome, the enzyme OPDA reductase 3 metabolizes the OPDA and dnOPDA to 3-oxo-2-pentenyl-cyclopentane-1-octanoic and hexanoic acid (Fig.1-11). These compounds undergo three cycles of β-oxidation using the enzymes ACX, MFP and thiolase to form JA and other JA derivatives (Fig.1-11). JA methyltransferases catalyze a methylation reation to produce MeJA in Arabidopsis (Seo et al., 2001; Delker. C. et al., 2007). JA-Ile is suggested to be the active form of JA under specific physiological conditions (Wasternack and Kombrink, 2010).

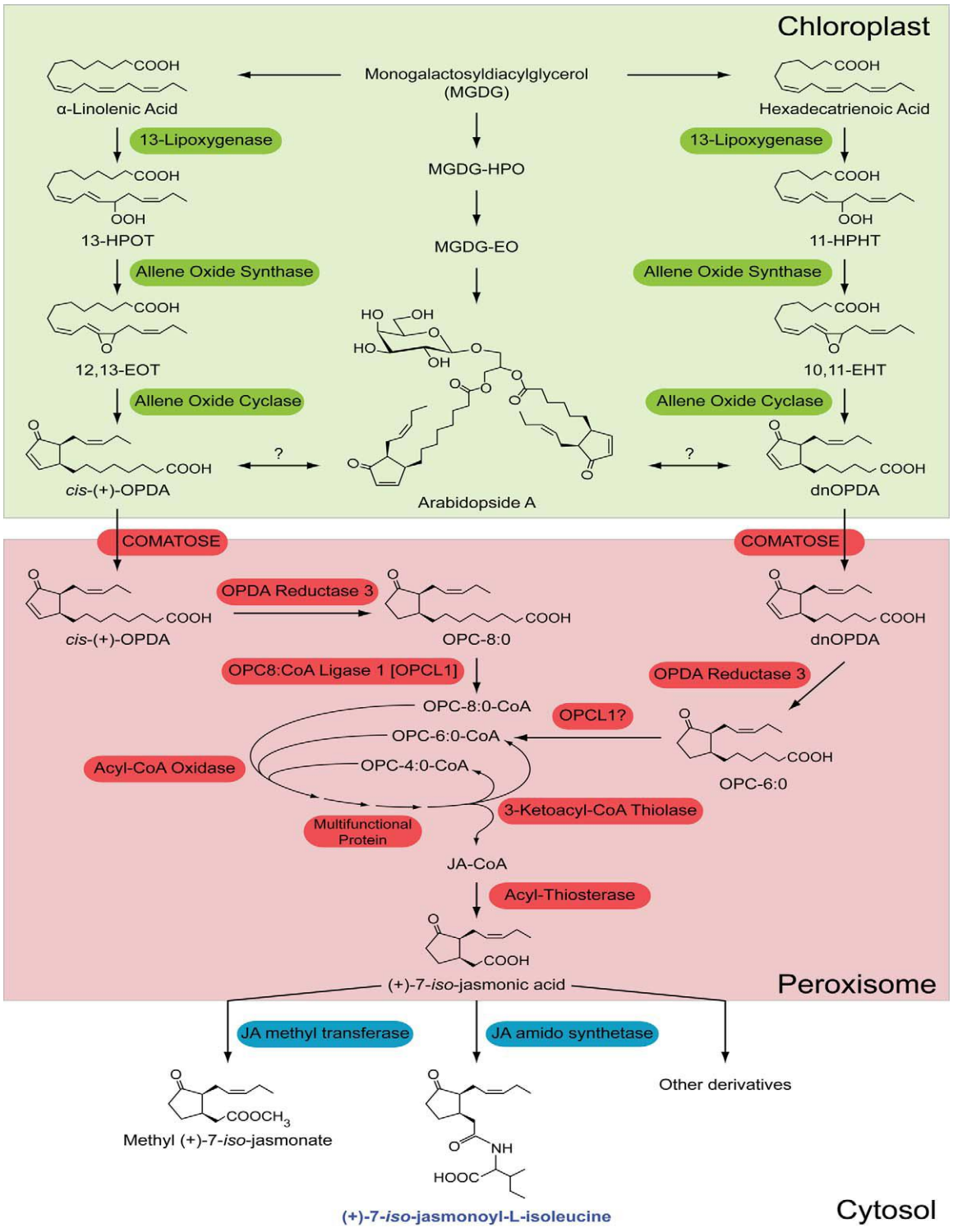


Figure 1-11. Jasmonic acid biogenesis in Arabidopsis (Taken from (Schaller and Stintzi, 2009; Acosta and Farmer, 2010).

1.2.c.iii. Jasmonate functions

(a) Jasmonate defense response in plants

In plants, JA plays an important role in activating defense responses (Degenhardt et al., 2010; Hind et al., 2010; Onkokesung et al., 2010; Yang et al., 2010) during abiotic and biotic stresses. Arabidopsis JA biosynthesis mutants have provided evidence of the critical importance of JA for defense response in plants (Browse, 2009; Degenhardt et al., 2010; Hind et al., 2010; Onkokesung et al., 2010; Yang et al., 2010). The Arabidopsis *opr3* mutant is compromised in 12-oxophytodienoate reductase (12-OPR) enzyme production, which is important for JA biosynthesis in the peroxisome (Stintzi and Browse, 2000). The *opr3* mutant displayed increased resistance to the insect *Bombus impatiens* and the fungus *Alternaria brassicicola* as compared to JA insensitive plants. *opr3* plants treated exogenously with OPDA are capable of robustly up-regulating the expression of a series of genes related to defense.

(b) Jasmonates in plant reproduction

Apart from their role in the physiological processes of survival, jasmonates also perform important roles related to plant reproduction. The presence of JA and MeJA has been shown in anthers and pollen of *Camellia* (Yamane et al., 1982). JA was thought to be the endogenous pollen germination regulator. However, two jasmonate compounds, *N*-[(-)-jasmonoyl]-(*S*)-isoleucine and *N*-[7-iso-cucurbinoyl]-(*S*)-isoleucine, were identified in pollen grains of *Pinus mugo* (Knöfel and Sembdner, 1995). It was shown that these jasmonates play an important role in maturation and germination of pollen grains. The role of jasmonates in floral reproduction was further shown when Arabidopsis mutants

incapable of synthesizing JA were shown to be male-sterile. Jasmonates are important not only for proper pollen development, but are also important for stamen elongation and the correct timing of pollen release (Liechti and Farmer, 2006; Avanci et al., 2010). *coi1* mutants were incapable of producing viable pollen grains and therefore were male-sterile (Feys, 1994). Genes encoding enzymes of the jasmonate biosynthetic pathway were shown to be specifically expressed in floral organs. Such floral organ-specific expression suggests that signals produced in these organs are transported to the stomium, the tissue responsible for the release of mature pollen grains (Cheong and Choi, 2003).

The gene *DDE1* (*Delayed dehiscence 1)* encodes the enzyme 12-oxophytodienoate reductase and *dde1* mutants exhibited delayed dehiscence of the anther, resulting in inefficient fertilization. Application of exogenous JA restored the mutant to the wild-type phenotype and this male-sterile mutant produced seeds. Additional Arabidopsis mutants with a male-sterile phenotype were shown to occur in enzymes responsible for the conversion of α-LeA to JA, important steps of the jasmonate biosynthesis pathway (Stintzi and Browse, 2000; von Malek et al., 2002). One of the mutations was shown to occur in a gene for 12-OPR, *OPR3* (Stintzi and Browse, 2000). *opr3* plants were sterile but were rescued by the application of exogenous JA, but not OPDA. Therefore, it was suggested that JA is the signaling compound responsible for the induction and coordination of anther filament elongation and the production of viable pollen in Arabidopsis (Stintzi and Browse, 2000). *opr3* stamens were treated with JA and used for gene expression analysis. Thirteen transcription factors (TFs) were identified; these TFs

such as MYB21 and MYB24 are involved in stamen maturation, are JA-inducible, and are important in stamen development (Mandaokar et al., 2006).

Although *dde2-2* mutants are defective in anther filament elongation, anther dehiscence, and are male-sterile but application of MeJA rescued these mutants to a wild-type phenotype. Additional studies of gene expression and complementation analysis showed that *dde2-2* male sterility was caused by interruption of the gene sequence encoding AOS, one of the main enzymes of the JA biosynthesis pathway (von Malek et al., 2002).

In *Arabidopsis,* ACX1 has been shown to play a role in wound-induced JA synthesis (Cruz Castillo et al., 2004; Li et al., 2005; Pinfield-Wells et al., 2005; Schilmiller et al., 2007). Accumulation of JA in the *acx1-1* mutant was 13% of wild-type (Pinfield-Wells et al., 2005), whereas in antisense *ACX1* plants JA accumulation were 50 – 60% of Wt (Cruz Castillo et al., 2004). In wounded T-DNA knockout *acx1* plants, JA accumulation was 20% of Wt (Schilmiller et al., 2007). In contrast, in wounded *acx5* plants JA accumulation was greater than Wt by 1.5 fold (Schilmiller et al., 2007). However, JA accumulation was 1% of Wt in wounded *acx1acx5* plants (Schilmiller et al., 2007). JA is also involved in male fertility in *Arabidopsis* (Browse, 2005; Schilmiller et al., 2007) and *acx1acx5* produces fewer siliques and viable seeds than wild type. However, this phenotype was rescued when flowers were treated with JA (Schilmiller et al., 2007). These results suggest that ACX1 is responsible for the majority of JA synthesis in *Arabidopsis,* although ACX5 may play a redundant role in JA biosynthesis, which can be observed in the absence of ACX1 (Schilmiller et al., 2007).

1.2.d. Photorespiration

The process of photorespiration occurs when the photosynthetic enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) oxidizes ribulose-1-,5-bisphosphate to generate 3-phosphoglycerate and phosphoglycolate (MacAdam, 2009; Bauwe et al., 2010). Photorespiration is considered a useless process since it reduces the photosynthetic efficiency of a plant (Maurino and Peterhansel, 2010). During this process, carbon is oxidized instead of reduced as in this case of photosynthesis. The 3-phosphoglycerate is resynthesized to ribulose bisphosphate and the phosphoglycolate is reduced (MacAdam, 2009). Thus, the 3-phosphoglycerate is targeted to the Calvin-Benson cycle, while the phosphoglycolate is recycled to the peroxisome (MacAdam, 2009; Bauwe et al., 2010). The phosphoglycolate is then transferred from the peroxisome to the chloroplast where it is dephosphorylated to glycolate (Foyer et al., 2009). The glycolate is transferred to the peroxisome where it is oxidized to glyoxylate and hydrogen peroxide is released as a by-product (Fig. 1-12). In the peroxisome, glyoxylate is converted to the amino acid glycine (Fig.1-12). The glycines are targeted to the mitochondria where two glycines form one serine and carbon dioxide and ammonia are released (Fig. 1-12). The serine is again transferred to the peroxisome to be deaminated to glycerate (Fig. 1-12). In the final step, the glycerate is again targeted to the chloroplast to be phosphorylated to 3-phosphoglycerate and then to the Calvin-Benson cycle (Fig.1-12).

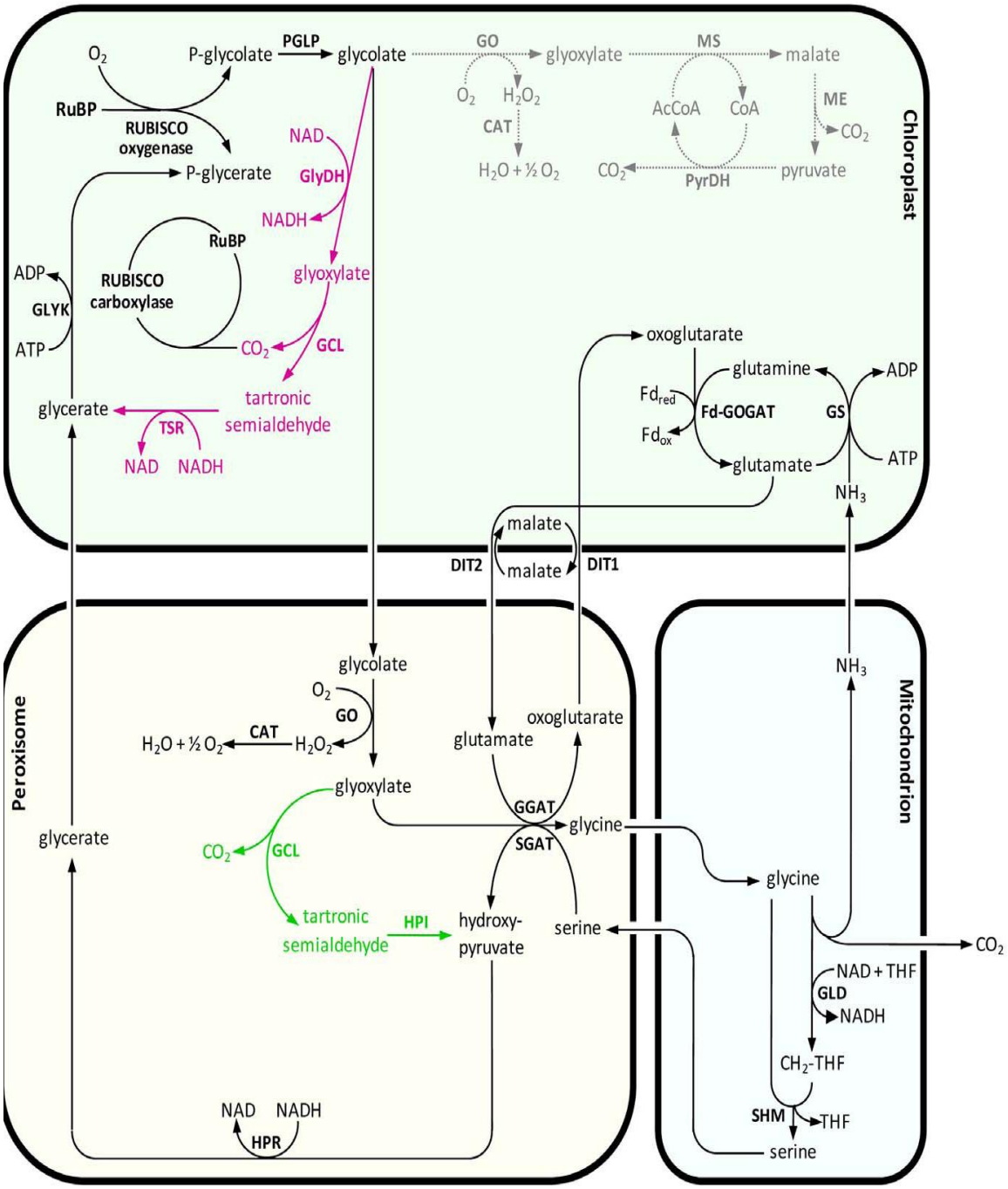


Figure 1-12. Role of peroxisomes in photorespiration (Peterhansel et al., 2010).

Studies have shown that photorespiration is important for removing toxic intermediates from cells. Phosphoglycolate was shown to inhibit triose phosphate isomerase and chloroplast phosphofructokinase (Anderson, 1971; Kelly and Latzko, 1976). Thus, due to these inhibitory effects, accumulation of phosphoglycolate in Arabidopsis mutants resulted in plants with reduced photosynthetic efficiency (Sommerville and Ogren, 1982; Maurino and Peterhansel, 2010). Glyoxylate, another compound of the photorespiratory pathway, also reduced photosynthetic efficiency by inhibiting the activation of Rubisco (Campbell and Ogren, 1990a; 1990b). Other studies showed that the enzyme glyoxylate reductase, present in the chloroplast and cytosol, prevents excess glyoxylate from reducing photosynthesis (Givan and Kleczkowski, 1992; Allan et al., 2009). Thus, photorespiration is important for removing accumulation of toxic intermediates from the cell that might otherwise reduce the photosynthetic efficiency by converting them to useful compounds. Photorespiration is an important pathway for the generation of hydrogen peroxide. Hydrogen peroxide is an important signaling molecule for defense response in plants by strengthening the cell wall and inducing the synthesis of defense molecules (Wu et al., 1997). Hydrogen peroxide was also shown to directly trigger programmed cell death in plants (Chamnongpol et al., 1998; Heath, 2000; Yoda et al., 2009; Chaouch et al., 2010). Compounds generated by the photorespiratory pathway are also important for primary metabolism. This pathway synthesized amino acids, glycine and serine, that can be used to synthesize other compounds (Mouillon et al., 1999; Novitskaya et al., 2002; Foyer et al., 2003; Engel et al., 2007; Peterhansel et al., 2010). Because this pathway forms a connection between the chloroplast, peroxisome and

mitochondria, it is used to transport information about the energetic state of these organelles (Nunes-Nesi et al., 2008).

1.3. OVERVIEW OF STUDY

In this work, we use mutants in the model organism *Arabidopsis thaliana*, a weed in the mustard family, to study peroxisome import and processes. Arabidopsis has five chromosomes with about 29,388 genes and a genome size of ~120 Mb. Arabidopsis is widely used for genetic studies because it is easy to manipulate in the lab and it has a short life cycle (8 weeks). Arabidopsis, like other oil seed plants, stores fatty acids in the seeds, and these fatty acids are β-oxidized to provide energy for germination and early seedling development. The processes described in this study occur in the peroxisomes.

In this study, I took a genetic approach to better understand the ACX enzymes fatty acid substrate specificity and to the role/s of each ACX in overall plant growth and development. Acyl CoA oxidases (ACX) catalyze the first step in peroxisomal fatty acid β-oxidation. There are six *ACX* genes in Arabidopsis, but only *ACX1-ACX5* are expressed. *In vitro* biochemical assays showed that the ACX enzymes have specific, but overlapping fatty acid chain length specificity. One previous study also showed that an *acx3acx4* double mutant in Arabidopsis accession Wassilewskija (Ws) is embryo lethal (Rylott et al., 2003), indicating a role for ACXs or fatty acid substrates/products in embryogenesis. The latter part of the jasmonic acid synthesis pathway takes place in peroxisomes, and ACX1 and ACX5 play a role in jasmonic acid synthesis.

I generated various combinations of *acx* double and triple mutants in Arabidopsis accession Columbia-0 (Col-0). I also generated *acx* mixed double mutants with Arabidopsis accessions Col-0 and Ws. In chapter 3, I set out to determine whether there were differences in the *ACX* genes in different Arabidopsis accessions. I focused mainly on the *acx* single mutants in Col-0 and Ws and an *acx1acx3acx4* triple mutant in Col-0 accession. I conducted biochemical assays to determine the roles of ACX1, ACX3 and ACX4 protein *in planta*, to look at the degree of overlap. I also sequenced the *ACX* genes and conducted phenotypic assays to determine what differences in ACX1, ACX3 or ACX4 accounted for the viability of the *acx1acx3acx4* in the Col-0 accession, while the *acx3acx4* in the Ws accession is embryo lethal. In Chapter 4, I conducted biochemical and phenotypic assays with all additional combinations of *acx* double and triple mutants to elucidate the role/s of ACX2 and ACX5 in overall plant growth and development.

Peroxisomes lack genetic material, so proteins that work in the peroxisome matrix are imported posttranslationally. PEX5 and PEX7 are the two receptors that import proteins into the peroxisome matrix. Proteins destined for the matrix are tagged with one of two peroxisome targeting signals (PTSs). Chapter 5 of this work described mutants in the *PEX5* receptor that specifically disrupt PTS1, PTS2 or both PTS1 and PTS2 matrix protein import. The *pex5* mutants were used to better understand peroxisome matrix protein import and the processes that are affected by blocking the import of PTS1 or PTS2 proteins.

To date, 30 PEX proteins have been identified in yeast while only 16 PEX proteins have been identified and characterized in plants. Thus, the mechanism of peroxisome import and recycling in plants has not been completely elucidated. Further, the enzymes involved in various peroxisome processes in plants is still unknown. Therefore, it is important to continue screening for peroxisome mutants in order to elucidate these processes. In chapter 6, I conducted a screen with ethyl methanesulfonate (EMS) mutagenized Ws seeds to isolate and characterize additional peroxisome mutants since the screen for peroxisome mutants is not saturated.

Taken together, this study will help in elucidating the mechanism of peroxisome import and the proteins (PTS1 or PTS2) that work in specific peroxisome processes. It will also provide information about the *in planta* roles of the ACX enzymes in peroxisomal β-oxidation processes.

CHAPTER 2: Materials and Methods

2.1. Plant materials

Arabidopsis thaliana accessions Columbia (Col-0) and Wassilewskija (Ws) were used in these studies.

2.1.a. Mutant isolation and genotyping

All insertion mutants contain a T-DNA insert in the respective gene. *acx1-2* (Salk_041464; At4g16760), *acx2-1* (Salk_006486; At5g6511), *acx3-6* (Salk_044956; At1g06290), *acx4-3* (Salk_065013; At3g51840), *acx4-1* (Salk_000879; At3g51840); *acx5-1* (Salk_009998; At2g35690), *acx1-2 acx3-6*, *acx1-2 acx5-1* were characterized previously ((Adham et al., 2005). All these mutants are in Col-0 accession. Various combinations of the *acx* single mutants were crossed to generate double mutants and double mutants were crossed to generate triple mutants (Table 2-1). Mixed *acx* double mutants were generated by crossing *acx* mutants from different accessions (Table 2-1). *chy1-3* (At5g65940) (Zolman et al., 2001), *aux1-7* (At2g38120) (Pickett et al., 1990) and *pex6-1* (At1g03000) (Zolman and Bartel, 2004) were used as controls. *pex5-1* has been previously published with a Ser-to-Leu mutation at amino acid 318 in At5g56290 (Zolman et al., 2000). *pex5-10* was obtained from the SALK collection of insertion mutants (SALK_124577). All mutants were backcrossed at least twice prior to our analyses. For all assays, *pex5-10* seeds were nicked. *acx3-1*, *acx3-2* and $acx4_W$ are in Ws accession.

Insertion mutants were genotyped with gene specific and T-DNA primers (Table 2-2), as indicated in Table 2-3. *acx3-1*, *acx3-2, pex6-1*and *pex5-1* single base-pair point mutants

were screened by PCR amplification with primers surrounding the mutation (Table 2-2) followed by restriction digestion with the appropriate restriction enzymes (Table 2-4). Double and triple mutants were genotyped with the combination of single mutant primers (Table 2-2). For the *acx3-2acx4-1* mixed double mutant, the background of the *ACX1* and *ACX2* genes was determined by PCR amplification with primers surrounding a polymorphism followed by restriction digestion with the appropriate restriction enzymes (Table 2-4). Primer sequences and primer combinations for genotyping mutants and transgenics are in Table 2-2 and Table 2-3, respectively. PCR program R-55 was used to genotype all mutants, except for the GFP mutant lines when the R-56 program was used. PCR program conditions are listed in Table 2-5.

Table 2-1. Crosses to generate *ACX* double and triple mutants.

Crosses	Mutants
acx1-2 X *acx2-1*	*acx1-2acx2-1*
acx1-2 X *acx3-6*	*acx1-2acx3-6*
acx1-2 X *acx4-3*	*acx1-2acx4-3*
acx1-2 X *acx5-1*	*acx1-2acx5-1*
acx2-1 X *acx3-6*	*acx2-1acx3-6*
acx2-1 X *acx4-3*	*acx2-1acx4-3*
acx2-1 X *acx5-1*	*acx2-1acx5-1*
acx3-6 X *acx4-3*	*acx3-6acx4-3*
acx3-6 X *acx5-1*	*acx3-6acx5-1*
acx4-3 X *acx5-1*	*acx4-3acx5-1*
acx1acx2 X *acx1acx3*	*acx1acx2acx3*
acx1acx2 X *acx1acx4*	*acx1acx2acx4*
acx1acx3 X *acx1acx4*	*acx1acx3acx4*
acx1acx2 X *acx1acx5*	*acx1acx2acx5*
acx1acx3 X *acx1acx5*	*acx1acx3acx5*
acx1acx4 X *acx1acx5*	*acx1acx4acx5*
acx2acx3 X *acx2acx4*	*acx2acx3acx4*
acx2acx3 X *acx2acx5*	*acx2acx3acx5*
acx2acx4 X *acx2acx5*	*acx2acx4acx5*
acx3acx4 X *acx3acx5*	*acx3acx4acx5*
Mixed double mutants	
Ws *acx3-2* X Col *acx4-1*	Ws *acx3-2*Col *acx4-1*
Ws *acx3-2* X Col *acx4-3*	Ws *acx3-2*Col *acx4-3*
Col *acx3-6* X Ws *acx4*	Col*acx3*-2Ws *acx4*

Table 2-2. Primer sequences.

Primer	Description	Sequence
ACX1-8 (a)	forward	5'-TGGTGGCCTGGTGGTTTGGG-3'
ACX1-5	reverse	5'-GAATTATATGCTCCATTTCCCATCTTTGTCCCG-3'
ACX1-20	forward	5'-TTCACGCTATTGTTTAGGTTGCTCG-3'
ACX1-25	reverse	5'-ATGAGCTGACATGGCAACCAATGG-3'
ACX1-17	forward	5'-CCGTCGTAACGGCCAAGAACCGAGTTCAA-3'
ACX1-16(a)	forward	5'-CTCCTCCACAAACATCTCGGCGATTTCCTC-3'
ACX1-13(a)	reverse	5'-CCGCCATATGACGATCGTAC-3'
ACX1-14	forward	5'-TTGAAGTCTCTCACCACCACAGCCAC-3'
ACX1-15	reverse	5'-GTACAACATCAGGGTTTAACCAATCCTCAG-3'
ACX1-17(a)	forward	5'-GTCCGGCCTAATGCGGTTGCACTTGTGGA-3'
ACX2-7	forward	5'-CCAACGATTGTCTTTACACCTATCTCCCTC-3'
ACX2-8	reverse	5'-GACATATCAACACTTCCAACAGCTTCCATG-3'
ACX2-9	reverse	5'-GCAACCGGTGTAATCAAGATTGTCAATGCC-3'
ACX2-18	forward	5'-CAATTCGGGCCTCCAAAGCAACCTGAGGTC-3'
ACX2-20	reverse	5'-CCTTCCCAACGCGCTGTAACGGGATTTGGCTG-3'
ACX3-2	forward	5'-TCCTCCATCTTCGAACCCGTCCCT-3'
ACX3-2(a)	reverse	5'-CCACAGGAAGAAATGCACACC-3'
ACX3-7	forward	5'-AACAAACCTTTCAAGGGACTGGGATTGGAG-3'
ACX3-8	reverse	5'-CGAGCTTACAACAGTTTCTGTAGATATTGTGG-3'
ACX3-17	forward	5'-GTGTTTTCACAGCTTCATATCAACGG-3'
ACX3-4	reverse	5'-CATTAGCTGTAACAGAGAAGGCTCTT-3'
ACX3-26	forward	5'-GACGTAAGGAGCTTTATAAACATTGTAGCC-3'
ACX3-25	reverse	5'-AATTCATTTGCAGCAAAACTCATAGCATAC-3'
ACX3-29	reverse	5;-GGGTCATAAGTTGTCACTGTTTCAATTCCCC-3'
ACX4-16(a)	forward	5'-GGGTTATGGATGTCCTGGTCTCTC-3'
ACX4-14	forward	5'-CTTGTGCAGATGCTGGGTAACGTTCAAGC-3'
ACX4-12	forward	5'-AGCATTTCCTCAAGCAACTCCAGCCTCTA-3'
ACX4-13(a)	reverse	5'-CTTCTCCTTCTGTGCTTCTGATCCACAGAG-3'

ACX4-11	reverse	5’- CTTTGCTACCAGAAAATCTGCTAGAA-3’
ACX4-10	forward	5’-AACATGTTGTCTGATTACATAGTCAGTACGC-3’
ACX4-4	reverse	5’-AGATTATTTGGCACAATTTGTAGATGTAAACC-3’
ACX4-20	forward	5’-CTGCTTCGCTAGGTCGGGAATTACTTGG-3’
ACX4-21	reverse	5’TAGAGACGGCTACGTGTAGCCGGTTTGAAACTCGCA ATACCCG-3’
ACX5-10	forward	5’-CTACGCACTCTATCTCCTTCAC-3’
ACX5-19	reverse	5’-GCTGACATGCAAATGCAATAGTACAACC-3’
ACX5new-9	forward	5’-GGGCAGAGCAAAACACCTTTTGCAATGCAG-3’
ACX5new-8	reverse	5’-CACTCCTTTTCCTTCAATATCTCCCTCTAC-3’
ACX1 Kpn1	forward	5'ATCGGTACCATGGAAGGAATTGATCACCTCGCC G3
ACX1 not1 *(gd)*	reverse	5’-GCGCGCTCT GCGGCCGC TCA GAG CCT AGC GGT ACG AAG TTG C-3’
ACX5 Kpn1	forward	5'-CAGGTACCATGGAGAGAGTTGATCACCTTGCCG-3'
ACX5 not1 *(gd)*	reverse	5’GCGCGCGAG GCGGCCGC TTA GAG TTT GGC AGA GCG G-3’
LB1-Salk	T-DNA	5’-CAAACCAGCGTGGACCGCTTGCTGC-3’
JL202	T-DNA	5’-CATTTTATAATAACGCTGCGGACATC-3’
PEX5-4	forward	5’-GCCTTTGATAGTAGTATCAGTCGGTCAGG-3’
PEX5-14	reverse	5’-GTGGCAAGTAAGACCCTAAAGTGAAC-3’
PEX5-3	forward	5’-GTCGTTGGCTGAATATTTTGTTCGGC-3’
PEX5-21	reverse	5’-GGATATCAAATGCGACTCAAACACTGATGAC-3’
F10O3-7	forward	5’-CAGACTTTACTGGCAAAAGCTGTGGCG-3’
F10O3-16	reverse	5’-GCTTGCTCCTATAATAAACAGATCCTGGG-3’
GusF	forward	5’-GTAACAAGAAAGGGATCTTCACTCGC-3’
35S-F3	forward	5’-ATGACGCACAATCCCACTATCCTTCG-3’
NosR	reverse	5’-TATGATAATCATCGCAAGACCGGCA-3’
PEX5-26	forward	5’-AGATATTGATGATGGGATACAAGCTCGAGG-3’
PEX5-30	reverse	5’-CCCATCTTGAGATAGTGTATGGGCAAGC-3’
PEX5 Sal	forward	5’-GCGTCGACTACTGATAGATTCAACGACGGTG-3’
PEX5 454	reverse	5’-

B/N		CGCCGGCGAGTATGTCAGAGAAGACGTAGACACC -3’
EIF4A	forward	5’-CAACAATGCCACCAGAAGCTCTTGAG-3’
EIF4A	reverse	5’-CACAGCAACAGAACCGACTTCTTTTC-3’
PEX5-32	forward	5’-GGGTCAGAGTTCTTCCGTGGTTTTCGC-3’
PEX5-33	reverse	5’-CTCCCCTATCGGCAAAAAAATGCCGGG-3’
PEX5-34	forward	5’-AGATATTGATGATGGGATACAAGCTCGAGG-3’
PEX5-30	reverse	5’-CCCATCTTGAGATAGTGTATGGGCAAGC-3’
PEX5-35	forward	5’-GCTTGCCCATACACTATCTCAAGATGGG-3’
PEX5-15	reverse	5’-GGCTGAAGCTTGCTTGACCTGATTCTC-3’
PEX5-36	forward	5’-GAGAATCAGGTCAAGCAAGCTTCAGCC-3’
PEX5-37	reverse	5’-GCATTTGCCCATGCATCAGCTGAACTATC-3’

Table 2-3. Primer combinations used in this study.

Mutant isolation	Genomic primers	Insertion primers
acx1	*ACX1-20* + *ACX1-25*	*ACX1-25* + LB1- salk
acx2	*ACX2-7* + *ACX2-9*	*ACX2-9* + LB1- salk
acx3	*ACX3-17* + *ACX3-4*	*ACX3-4* + LB1- salk
acx4	*ACX4-10* + *ACX4-11*	*ACX4-11* + LB1- salk
Ws *acx4*	*ACX4- 10* + *ACX4-4*	*ACX4-4* + JL202
acx5	*ACX5-9* + *ACX5-8*	*ACX5-8* + LB1- salk
pex5-10	*PEX5-3* + *PEX5-21*	*PEX5-21* + LB1- salk

mutants & transgenics	Primers
Ws *acx3-2*	*ACX3-26* + *ACX3-25*
ACX1 genotype	*ACX1-16(a)* + *ACX1-13(a)*
ACX2	*ACX2-18* + *ACX2-20*
pex5-1	*PEX5-4* + *PEX5-14*
pex6-1	*F10O3-7* + *F10O3-16*
$PEX5_{454}$	*PEX5-26* + *PEX5-30* for *PEX5* cDNA
pex5-10GUS	*pex5-10* primers; *GusF* + *NosR*
pex5-1($PEX5_{454}$)	*pex5-1* primers; $PEX5_{454}$ primers
pex5-10($PEX5_{454}$)	*pex5-10* primers; $PEX5_{454}$ primers
Col-0*(GFPPTS2)*	*35S-F* + *NosR*
Col-0*(GFPSKL)*	*35S-F* + *NosR*
pex5-1(GFPPTS2)	*pex5-1* primers; *35S-F* + *NosR*
pex5-1(GFPSKL)	*pex5-1* primers; *35S-F* + *NosR*

pex5-10(GFPPTS2)	*pex5-10* primers; *35S-F + NosR*
pex5-10(GFPSKL)	*pex5-10* primers; *35S-F + NosR*
pex5-1 (PEX5$_{454}$)(GFPPTS2)	*pex5-1* primers*; PEX5$_{454}$* primers; *35S-F + NosR*
pex5-1 (PEX5$_{454}$)(GFPSKL)	*pex5-1* primers*; PEX5$_{454}$* primers; *35S-F + NosR*
pex5-10 (*PEX5$_{454}$*)(*GFPPTS2)*	*pex5-10* primers*; PEX5$_{454}$* primers; *35S-F + NosR*
pex5-10 (*PEX5$_{454}$*)(*GFPSKL)*	*pex5-10* primers*; PEX5$_{454}$* primers; *35S-F + NosR*

Primer combination for RT-PCR

ACX1-upstream	*ACX1-8(a) + ACX1-5*
ACX1-spanning	*ACX1-14 + ACX1-15*
ACX1-downstream	*ACX1-16(a) + ACX-17*
ACX2 – spanning	*ACX2-7 + ACX2-8*
ACX3-upstream	*ACX3-2 + ACX3-29*
ACX3 – spanning	*ACX3-17 + ACX3-4*
ACX3 – downstream	*ACX3-7 + ACX3-8*
ACX4 –upstream	*ACX4-16(a) + ACX4-13(a)*
ACX4 – spanning	*ACX4*-14 + *ACX4*-11
ACX4 – downstream	*ACX4*-14 + *ACX4*-11
ACX5 – spanning	*ACX4-20 + ACX4-21*
PEX5 – exon3+exon4	*PEX5-32 + PEX5-33*
PEX5 – exon5+exon6	*PEX5-34 + PEX5-30*
PEX5 – exon6+exon7	*PEX5-35 + PEX5-15*
PEX5 – exon7+exon8	*PEX5-36 + PEX5-37*
PEX5 – exon3+exon7	*PEX5-32 + PEX5-15*

Probes for northern blot	
ACX1 – probe	*ACX1-17(a) + ACX1-13(a)*
ACX2 – probe	*ACX2-7 + ACX2-8*
ACX3 – probe	*ACX3-2 + ACX3-2(a)*
ACX4 – probe	*ACX4-14 + ACX4-4*
ACX5 – probe	*ACX5-10 + ACX5-19*

Table 2-4. Genotyping mutants.

Mutant	Restriction enzyme	Products (bp)
pex5-1	*Eco*RI	Wt = 216, 226, 120 Mut = 342, 126
pex6-1	*Xho*I	Wt = 300, 120 Mut = 420
acx3-2	*Ecor*V	Wt = 171 Mut = 196
acx3-2acx4-1		
ACX1	*Hae*III	Col = 300, 150, 72 Ws = 300, 225
	RsaI	Col = 350, 210 Ws = 560
gfppts2	*Nco*I	~500, ~300
gfpskl	*Hind*III	~900

Table 2-5. PCR programs used.

Program	Number of cycles	Denaturation		Annealing		Elongation	
		time	temp (^{0}C)	time	temp(^{0}C)	time	temp(^{0}C)
R-55	40	15 sec	95	30 sec	55	1 min	72
R-56	40	30 sec	95	30 sec	55	3 min	72

2.2. Phenotypic assays

Seeds were surface sterilized with 30% bleach solution for 10 min, rinsed four times with sterile water and plated on solid plant nutrient media (PN) (Haughn and Somerville, 1986), supplemented with 0.5% sucrose (PNS), alone or with the indicated hormone. All assays were conducted at least three times with similar results. For experiments with pex5 (chapter 5), immediately after plating, all *pex5-10* seeds were nicked under a Leica Zoom 2000 dissecting microscope, unless otherwise indicated.

2.2.a. Root elongation

For root elongation assays, seeds were plated on PNS alone or with the indicated concentration of indole-3-butyric acid (IBA), 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB), indole acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), jasmonic acid (JA), isobutyrate or propionate. All plates were wrapped with gas permeable micropore surgical tape and incubated for the indicated time at 22^{o}C; if plates contained auxins, yellow-filtered light was used. Seedlings were removed from plates and primary root length was measured in millimeters (mm).

2.2.b. Lateral root initiation

To determine lateral root initiation, seeds were plated on PNS. After 5 days, seedlings were transferred to fresh PNS plates supplemented with or without hormone and incubated under yellow filters for 5 additional days. The number of lateral roots was counted with a Leica Zoom 2000 dissecting microscope and the root length measured. Data are represented as lateral root density: lateral root number/root length (mm).

2.2.c. Sucrose dependent assays

To assay sucrose dependence in the dark, seeds were plated on PN medium alone or on medium supplemented with the indicated concentrations of sucrose. Plates were incubated at 22^{o}C under continuous white light for 24 h then transferred to dark for the indicated number of days, after which the length of the hypocotyls was measured. For sucrose dependence assays in the light, seeds were plated on PN alone or supplemented with the indicated concentrations of sucrose and incubated at 22^{o}C under continuous white light for 7 days, then root length was measured.

2.2.d. Germination assays

For germination assays, 100 seeds were plated on PN supplemented with or without 0.5% sucrose. F_2 seeds were the second (segregating) generation of a back-cross to the Col-0 parent. Seeds were incubated at 22^{o}C under continuous white light for 24 h. Then plates were examined under a dissecting microscope for radicle emergence and the number of seeds that germinated were marked every 24 hr for 4 d. Seeds that did not germinate by day 4 were transferred in a sterile manner to a fresh PNS plate and the seed coats were nicked with forceps under a dissecting microscope. After 10 days, all the seedlings were collected and DNA was extracted from individual seedlings. The genotype of each seedling was determined by PCR amplification of the extracted DNA with gene-specific *PEX5* primers and T-DNA primers (Table 2-2).

For germination assays with homozygous *pex5-10* and *acx* mutants, 100 seeds were plated on media with 0.5% sucrose. Seeds were incubated at 22^{o}C under continuous

white light for 24 hr, then plates were examined under a dissecting microscope for radicle emergence and the number of seeds that germinated were marked every 24 hr for 4 d.

2.2.e. Chlorophyll quantification

Chlorophyll extraction and quantification were performed using modifications to a previous protocol (Wintermans and Demots, 1965). 5 mg of 8 day old seedlings or 4 week old leaves was boiled in 96% ethanol at 80^{o}C until all the chlorophyll was removed from the leaf. The chlorophyll content was then measured with a spectrophotometer at OD654, OD665 and OD649 and total chlorophyll content was calculated according to the previous protocol (Wintermans and Demots, 1965).

2.2.f. DAB and DR5-GUS staining

To visualize accumulation of H_2O_2, 3,3'-Diaminobenzidine tetrahydrochloride (DAB) stain was used. DAB reacts with H_2O_2 to produce a brown precipitate. A modified version of the (Thordal-Christensen et al., 1997) protocol was used for staining. Briefly, leaves were collected from two weeks old plants and vacuum infiltrated with 1mg/ml DAB stain solubilized in tris-acetate buffer, pH 3. Following infiltration, leaves were incubated with the stain for 24 hr in the dark, then boiled in 80% ethanol three times to remove chlorophyll for visualization.

For localization of DR5-GUS, Col-0 and *pex5-10* transgenic seedlings were stained at 37^{o}C for 2 days in 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as previously described by (Bartel and Fink, 1994). Seedlings were mounted on slides and pictures

were taken under a microscope. DR5-GUS is a construct where the expression of the *GUS* gene is driven by an auxin inducible promoter, *DR5*.

2.2.g. Adult phenotypic analysis

For the adult phenotypic assays, seeds were plated on solid PNS medium. Seeds were then incubated at 22^{o}C under continuous white light for 10 days. Seedlings were then transferred to pots of Sun-Gro Metro Mix 360 soil and incubated at 22^{o}C – 25^{o}C under continuous white light. Plants were watered once weekly and measurements were taken at regular intervals for rosette diameter, plant height and number of stems. Each plant was observed daily for date of bolting. The 5th silique from the main stem was measured for each plant. The weight of 100 wild-type and mutant seeds was recorded.

2.2.h. Constructing a PTS1 specific mutant

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

I created a truncated gene construct of the first 454 amino acids of the PEX5 protein. The truncated *PEX5* cDNA was amplified with primers PEX5-*Sal* and PEX5 454 B/N (Table 2-2), the PCR product was electrophoresed on a 1% guanosine gel and the DNA was purified with Qiaex II Gel extraction kit (Qiagen). The purified product was ligated into a TOPO vector and the insert was verified by sequencing prior to subcloning into pBlueScript vector (pKS). For myc ligation to $PEX5_{454}$, pT26 myc and pKS PEX5 454

were digested with *Bam*HI and the pT26myc. The digestion product was electrophoresed on a 1% guanosine gel and the myc fragment was extracted. The *Bam*HI digested pKS *PEX5*$_{454}$was dephosphorylated and ligated to myc and then transformed into *E. coli* DH5α cells (Invitrogen). For cloning of the *PEX5*$_{454}$ insert (with the myc epitope) into the plant transformation vector 35S pBARN (LeClere and Bartel, 2001), pKS PEX5$_{454}$ was digested with *Apa*I and a blunt end was created followed by a *Not*I digest. The digested product was electrophoresed on a 1% gel and the *PEX5*$_{454}$ DNA was extracted with Perfect-prep Gel cleanup kit (Eppendorf). 35SpBARN was digested with *Sma*I and *Not*I and PEX5$_{454}$ was ligated to this vector and transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). This construct was transformed into wild-type plants (Bent and Clough, 1998), which were selected by Basta resistance and both native and truncated PEX5 levels were confirmed by immunoblot. This Col-0 (*PEX5*$_{454}$) line then was crossed to *pex5-1* and *pex5-10*, which were selected first by genotyping the mutation (Table 2-2; Table 2-3), then by identifying lines containing the cDNA (Table 2-2; Table 2-3).

2.2.i. Sequencing

DNA was extracted from Ws seedlings and *ACX1*, *ACX3* and *ACX4* genes were PCR amplified with different combinations of primers. DNA was extracted from *pex5-10* seedlings and the *PEX5* gene was PCR amplified with various primer combinations. The R-56 PCR program was used for amplification of *ACX1*, *ACX3*, *ACX4* and *PEX5* genes (Table 2-5). PCR products were electrophoresed on a 1% agarose gel and purified with the EZ gel extraction kit (EZ BioResearch) according to the manufacturer's

instructions. The purified DNA was send to the University of Missouri-Columbia DNA core facility for sequencing. Sequencing results for *ACX1*, *ACX3* and *ACX4* genes in Ws were compared to the *ACX1*, *ACX3* and *ACX4* genes from Col-0. The sequence result for the *PEX5* gene in *pex5-10* plants was compared to Wt Col-0 *PEX5* gene sequence.

2.3. Expression assays

2.3.a. mRNA isolation and cDNA synthesis

For RT-PCR, total RNA was isolated with the RNeasy Plant Kit (Qiagen) from Wt and mutant seedlings and plants. RNA was treated with DNase (Fisher Scientific) to remove contaminating genomic DNA and cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

For northern analysis, total RNA was isolated from 4 d old seedlings using a hot phenol-chloroform extraction method (Verwoerd et al., 1989).

2.3.b. Reverse transcriptase-PCR

For PCR amplification, 1µl of cDNA was used for PCR amplification with gene-specific primers located at the indicated positions on the genes. The primers sequence and combinations are shown in Tables 2-2 and Table 2-3. For all PCR amplifications, Col-0 genomic DNA was used as control. The R-55 PCR program was used (Table 2-5) and products were resolved on a 3% agarose gel.

2.3.c. Northern blots

For northern blot analysis, 30 µg of total RNA was electrophoresed on a formaldehyde gel and stained with ethidium bromide to check for equal loading and purity of the RNA. RNA was transferred to a nitrocellulose membrane overnight and UV cross-linked. Gene-specific probes were generated by PCR amplification of 300-400 bp regions from *ACX1*, *ACX2*, *ACX3*, *ACX4* and *ACX5* cDNAs. Primers for gene-specific probe amplification are shown in Tables 2-2 and Table 2-3. Ws and Col-0 sequences were compared in this region to ensure high sequence similarity in the different backgrounds. Probes were radio-labeled with ^{32}P-ATP using a random hexamer labeling protocol (Feinberg and Vogelstein, 1983) and blots were hybridized overnight at 42^{0}C. Blots were exposed for 3 d to a phospho-screen and detected with a phosphorimager. To quantify expression levels, blots were stripped and hybridized with a gene-specific *EIF4A* probe (At3g13920) generated in the same manner. Expression levels were calculated with an ImageQuant scanner.

2.4. Protein purification and assays

2.4.a. Protein purification.

For western blot analysis, seedlings or adult plants were ground on dry ice and mixed with 2X NuPAGE LDS Sample Buffer (Invitrogen). The mixture was centrifuged and the supernatant containing the protein was collected. Protein was quantified with Bradford reagent (Sigma-Aldrich).

2.4.b. Immunoblot analysis.

40 µg of total protein was electrophoresed on NuPAGE 10% Bis-Tris gel with 1X NuPAGE MOPS SDS running buffer (Invitrogen). Proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences) with NuPAGE transfer buffer (Invitrogen). Membranes were blocked for 2 hr in 10% (w/v) non-fat dry milk in TBS-T buffer (Ausubel et al., 1999). After blocking, membranes were incubated with primary antibodies recognizing the C-terminal region of PEX5 (Zolman and Bartel, 2004); 1:1000 dilution), the myc epitope (Sigma; 1:1000 dilution), or thiolase (Lingard and Bartel, 2009); 1:5000). An HSC70 antibody was used as a loading control (Stressgen Bioreagents; 1:5000). Secondary detection involved horseradish peroxidase-linked goat anti-rabbit antibodies (PEX5; Southern Biotech; 1:10000) and rabbit anti-mouse antibodies (myc and HSC70; Sigma; 1:10000). All antibodies were visualized using SuperSignal West Dura extended duration substrate (Thermo Scientific).

2.5. GFP localization.

To determine PTS1 and PTS2 localization in Wt and peroxisome mutants, GFP constructs were created with these signals. pBICaMVGFP, pBICaMVPTS2 and pBICaMVSKL were previously made and transformed into Col-0 plants by Dr. Zolman. The Col-0(*GFP)*, Col-0(*GFPPTS2)* and Col-0(*GFPSKL)* plants were crossed to *pex5-1*, *pex5-1(PEX5$_{454}$)*, *pex5-10*, *pex5-10(PEX5$_{454}$)* plants. Plants were genotyped (Table 2-2 and Table 2-3) and homozygous *pex5-1GFP, pex5-1GFPPTS2, pex5-1GFPSKL*, *pex5-1(PEX5$_{454}$)GFP*, *pex5-1(PEX5$_{454}$)GFPPTS2, pex5-1(PEX5$_{454}$)GFPSKL, pex5-10GFP, pex5-10GFPPTS2, pex5-10GFPSKL, pex5-10(PEX5$_{454}$)GFP, pex5-*

10(PEX5$_{454}$)GFPPTS2 and *pex5-10(PEX5$_{454}$)GFPSKL* plants were isolated. Roots were mounted on slides and visualized under a Ziess confocal microscope for GFP localization.

2.6. Biochemical assays

2.6.a. ACX enzyme assays

Wt and mutant seeds were surface sterilized and plated on solid PNS medium covered with filter paper. Seeds were incubated at 22^{o}C under continuous white light for 3 days, then harvested and stored at -80^{o}C until needed. ACX assays were similar to previously described protocols (Hryb and Hogg, 1979; Gerhardt, 1987; Adham et al., 2005). 0.3g of seedlings was immersed in liquid nitrogen and ground to powder in a mortar. Ground seedlings were incubated for 15 min at 4^{o}C in 800 µl cold extraction buffer containing 50 mM KPO_4, 50 µM FAD and 0.01% triton X-100 supplemented with 10 µl protease inhibitor cocktail (Sigma). After incubation, extracts were centrifuged for 10 min at 13,200 rpm and the supernatant was loaded on quick spin protein columns (Roche) and processed according to manufacturer's instructions. For the enzymatic reaction, 200µl of extract was added to 200µl reaction mix (50mM KPO_4, 0.025% triton X-100, 50 mM p-hydroxybenzoic acid, 100 µg/mg BSA, 50µM FAD, 2 mM 4-aminoantipyrine, 110U horseradish peroxidase) and 125 µM C6:0, C12:0, C16:0 and C18:1 substrate (Sigma). The reaction was measured spectrophotometrically at 500 nm for H_2O_2 production and a standard H_2O_2 curve was created to calculate pmol of H_2O_2. For assays with 3 and 1.5 day old Col-0 and Ws seedlings, extracts were made as described previously with 0.27g of 3-day-old seedlings or 0.1g of 1.5 day old seedlings. For enzyme assays in the dark,

seeds were plated on filter paper on solid PN medium alone or supplemented with 0.5% sucrose and incubated at 22^{o}C under continuous white light for 24 h, then transferred to dark for 2 days. 0.24 g of seedlings was used for the enzyme assay. For embryogenesis enzyme assays, green siliques were dissected under a microscope and 200 green embryos were extracted. Embryos were ground in 200 µl extraction buffer and used for the assay.

2.7. GC analysis of C20:1 storage fatty acid.

Seeds were surface sterilized, plated on solid PNS media covered with filter paper, and incubated at 22^{o}C under continuous white light for 6 days. Fatty acid extraction and esterification was done similar to previous protocol (Devaiah, 2007). 100 or 50 6 d old seedlings were added to 2 ml of methanol containing 1.5% H_2SO_4 and 0.01% butylated hydroxytoluene (BHT). Prior to esterification, 50µM heptadecanoic acid (C17:0) was added to each sample as an internal standard and samples were incubated for 1 h at 90^{o}C. After incubation, 1 ml of sterile water and 1 ml of hexane was added and the samples were vortexed for 1 min. Samples were then centrifuged at 900 rpm for 2 min at RT and the upper phase (500µl) containing the fatty acid methyl esters (FAME) were placed in glass vials for analysis. FAMEs were analyzed by automated Shimadzu gas chromatograph (Shimadzu GC-17A) equipped with a silica capillary column (DB-5MS, 30 m, 0.25mm, 0.25 1m). The temperatures of the injector column and flame ionization detector were 220, 170 and 220^{o}C. C20:1 fatty acid levels were divided by total fatty acid levels to calculate percent C20:1 remaining in seedlings at 6 days.

CHAPTER 3: An Acyl-CoA oxidase (ACX) triple mutant, *acx1acx3acx4* reveals variation between Arabidopsis accessions.

Previous studies examined the phenotypes of several single and double *acx* mutants; in this study, I focused on higher-order *acx* mutants to better understand the role of ACX enzymes in fatty acid β-oxidation and to determine which ACX proteins are involved in other peroxisomal β-oxidation processes. An *acx1acx3acx4* triple mutant in Col-0 was created by crossing *acx1acx3* and *acx1acx4* double mutants. The *acx3acx4* double mutant was isolated by crossing *acx3-6* and *acx4-3* single mutants and the *acx1acx4* double mutant was isolated by crossing *acx1-2* and *acx4-3* single mutants. Dr. Zolman and Raquel Adham did the mutant crosses. Amanda Tulin, Anupama Vijayaraghavan and Tad Wood helped with mutant genotyping. I confirmed these mutants by PCR and RT-PCR. Below I described the results for the *acx1acx3acx4* mutant characterization. The manuscript for this work is currently in preparation.

3.1. Mutant isolation

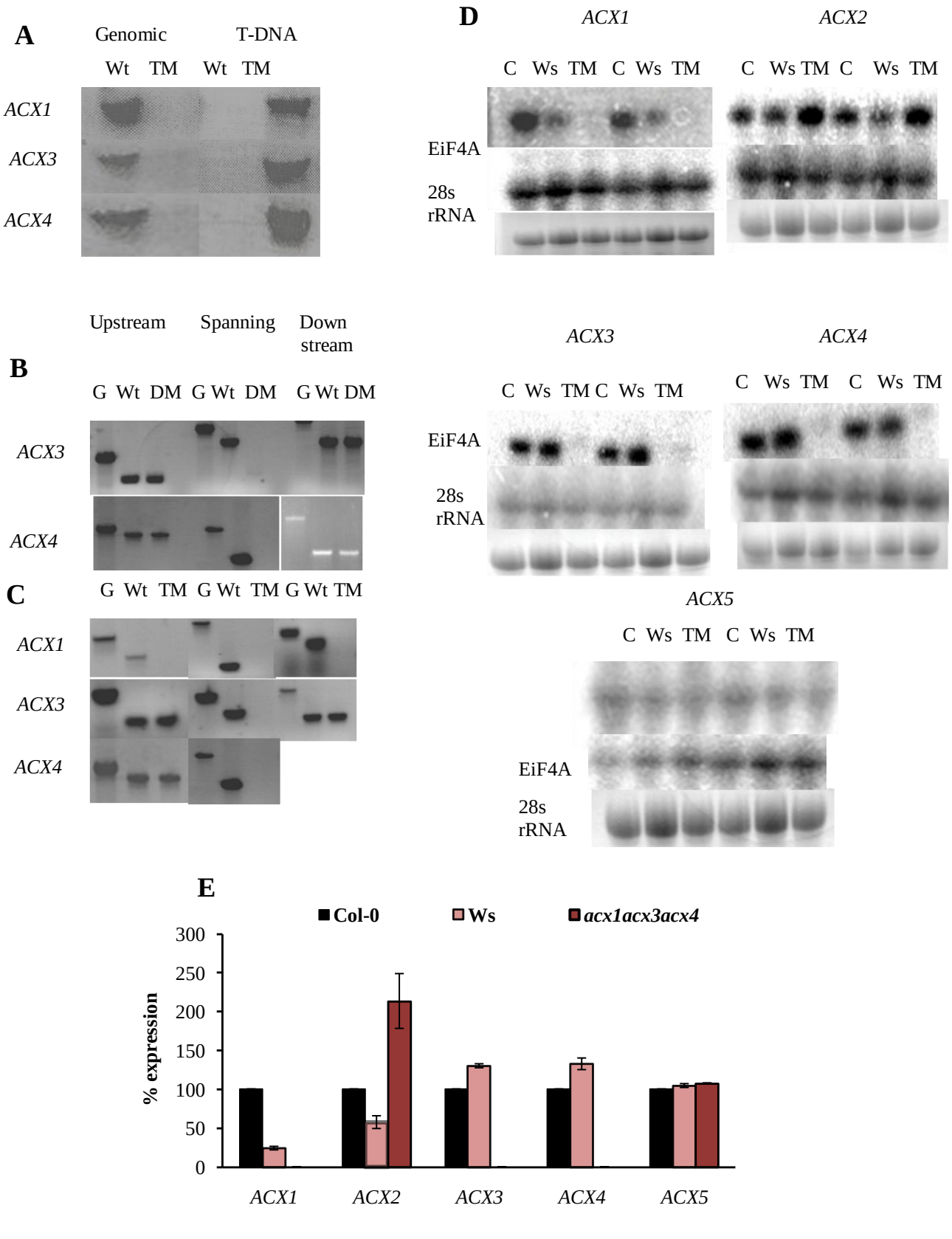
PCR analysis was used to genotype F_2 progeny to isolate the *acx3-6acx4-3* and *acx1-2acx3-6acx4-3* double and triple mutant. Genomic primers spanning the site of the T-DNA insertions in *ACX3* and *ACX4* amplified the expected products in Wt plants but failed to amplify any product in *acx3-6acx4-3;* likewise the *ACX1*, *ACX3* and *ACX4* spanning primers failed to amplify a product in *acx1-2acx3-6acx4-3* plants (Figure 3-1A). Amplification of the *acx3-6acx4-3* plants with *ACX3* and *ACX4* genomic primers 3' to

the T-DNA insert and the LB1Salk primer resulted in a product, while no product was amplified in Wt plants (Fig. 3-1A). Similarly, amplification with *ACX1*, *ACX3* and *ACX4* genomic primers 3' to the T-DNA insert and LB1Salk primer resulted in a product in *acx1-2acx3-6acx4-3* plants (Fig. 3-1A). This result suggests that homozygous *acx3-6acx4-3* and *acx1-2acx3-6acx4-3* mutants were successfully isolated.

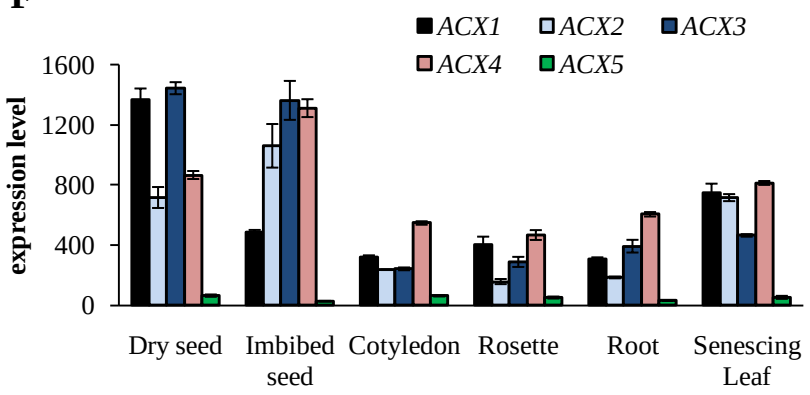
To confirm the *acx3acx4* mutant, RT-PCR was conducted on total RNA isolated from 8 d old seedlings. Primers upstream and downstream of the T-DNA insert in *acx3* and *acx4* amplified a product in Wt and cDNA of *acx3acx4* (Fig. 3-1B), while primers spanning the T-DNA insert in *acx3* and *acx4* failed of amplify a product in the *acx3acx4* mutant cDNA (Fig. 3-1B).

RT-PCR was performed on total RNA isolated from 8 d old Wt and *acx1-2acx3-6acx4-3* seedlings. Primers upstream of the T-DNA insert in *acx1*, *acx3* and *acx4* amplified the expected product in Wt and *acx1acx3acx4* seedlings (Fig. 3-1C). Primers spanning the sites of the T-DNA insert in *acx1*, *acx3* and *acx4* failed to amplify a product in the cDNA of *acx1acx3acx4* but amplified the expected product for Wt cDNA (Fig. 3-1C). *ACX3* and *ACX4* primers downstream of the T-DNA insert in *acx3* and *acx4* amplified the expected product size in the cDNAs of Wt and *acx1acx3acx4* (Fig. 3-1C). However, primers downstream of the T-DNA insert in *acx1* failed to amplify a product in the cDNA of *acx1acx3acx4* but amplified the expected product size in Wt cDNA (Fig.3-1C). These results suggest that truncated or modified transcripts of *ACX1*, *ACX3* and *ACX4* are produced in *acx1acx3acx4*.

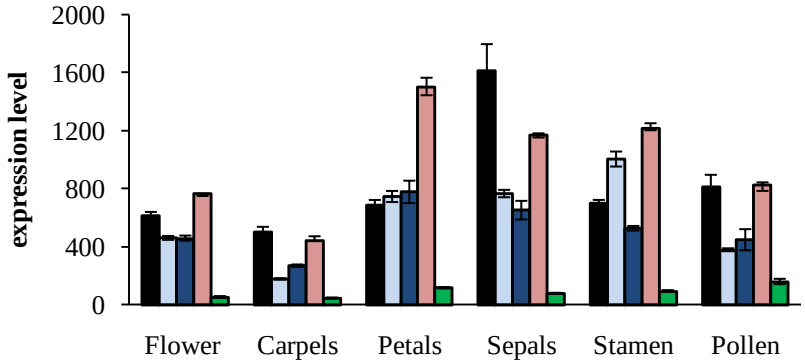
Northern blot analysis using gene-specific probes revealed that full-length products are not made in the triple mutant, although *ACX2* and *ACX5* gene expression remains intact (Fig. 3-1D, E). *ACX5* levels were similar in the triple mutant and wild type (Fig. 3-1D, E). However, *ACX2* levels were increased in *acx1acx3acx4* compared to Col-0 wild-type samples (Fig. 3-1D, E), indicating that *ACX2* gene expression may be responding to loss of the three *ACX* genes in the triple mutant. To determine whether acyl CoA oxidase genes expression is tissue specific, graphs of *ACX1*, *ACX2*, *ACX3*, *ACX4* and *ACX5* expression levels in different tissues and after treatment with MeJA were constructed from publicly available microarray data (Fig.3-1 F, G, H).


A
Genomic
T-DNA
Wt TM Wt TM
ACX1
ACX3
ACX4
B
Upstream
Spanning
Down stream
G Wt DM G Wt DM G Wt DM
ACX3
ACX4
C
G Wt TM G Wt TM G Wt TM
ACX1
ACX3
ACX4
D
ACX1
ACX2
C Ws TM C Ws TM
C Ws TM C Ws TM
EiF4A
28s rRNA
ACX3
ACX4
C Ws TM C Ws TM
C Ws TM C Ws TM
EiF4A
28s rRNA
ACX5
C Ws TM C Ws TM
EiF4A
28s rRNA
E
Col-0
Ws
acx1acx3acx4
% expression
300
250
200
150
100
50
0
ACX1
ACX2
ACX3
ACX4
ACX5

F


ACX1
ACX2
ACX3
ACX4
ACX5
expression level
1600
1200
800
400
0
Dry seed
Imbibed seed
Cotyledon
Rosette
Root
Senescing Leaf

G


expression level
2000
1600
1200
800
400
0
Flower
Carpels
Petals
Sepals
Stamen
Pollen

H

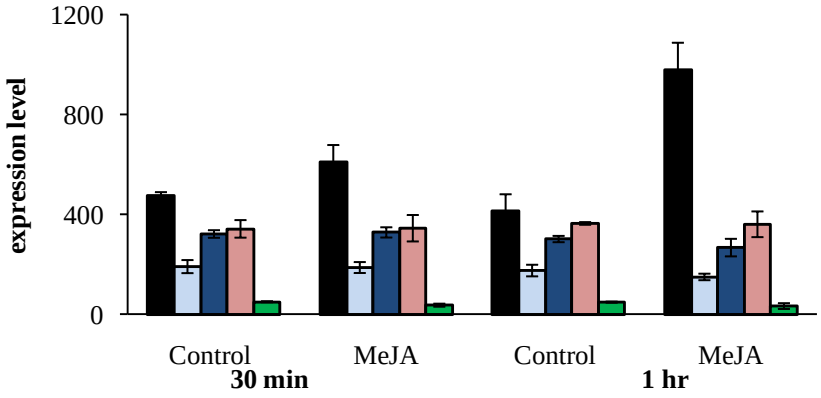

expression level
1200
800
400
0
Control
MeJA
Control
MeJA
30 min
1 hr

Figure 3-1. Confirmation of *acx3acx4* and *acx1acx3acx4* mutants
(A) DNA was extracted from progeny of a cross of *acx1-2acx3-6* and *acx1-2acx4-3* and PCR-amplified with *ACX1, ACX 3* and *ACX4* gene-specific and T-DNA primers. DNA from Wt Col-0 was used as a control with both set of primers in the PCR reactions.
(B, C) cDNA was synthesized from RNA isolated from 8 d old Col-0, *acx3acx4* (DM) and *acx1acx3acx4* (TM) seedlings. Col-0 and mutant cDNAs were amplified with *ACX1, ACX3* or *ACX4* specific primers upstream and spanning the site of T-DNA insertions. cDNAs were also amplified with *ACX, ACX3* and *ACX4* primers downstream of the T-DNA insertion sites. Genomic DNA (G) from Wt Col-o was amplified with all primer sets to show success of cDNA synthesis.
(D) Total RNA was isolated from Col-0, Ws, and *acx1acx3acx4* for northern blot analysis. Each blot was probed with labeled gene-specific PCR products amplified from Col-0 cDNA. *EiF4A* northern blots and rRNA from ethidium-bromide stained gels are shown as loading controls. Each blot contains two independent sets of samples and shows representative results.
(E) Expression levels from northern blots hybridized with *ACX* gene-specific probes or an *EiF4A* control probe were calculated using an ImageQuant scanner. Data are plotted as the average percent expression, with individual Col-0 values set at 100%. Values were calculated from 4 blots (*ACX1, ACX4*), 9 blots (*ACX2*), 6 blots (*ACX3*), and 2 blots (*ACX5*). For *ACX1-ACX4*, error bars represent the standard error of the mean.
(F) Levels of *ACX1-ACX5* mRNA expression in dry and imbibed seeds, seedling cotyledons, rosette leaves, roots, and senescing leaves.
(G) Levels of *ACX1-ACX5* mRNA expression in whole flowers, carpels, petals, sepals, stamen, and mature pollen. All flower values were from stage 15 tissue.
(H) Levels of *ACX1-ACX5* mRNA expression in wild-type seedlings treated with 10 µM Me-JA for 30 min or 3 h. Graphs from F, G, and H were constructed using publically-available microarray data from the *Arabidopsis* eFP Browser data at http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007). Data was retrieved on January 22, 2010.

3.2. Fatty acid β-oxidation in *acx1acx3acx4*

As described in section 1.2.a., fatty acid β-oxidation efficiency can be measured indirectly by examining growth on media without sucrose; wild-type plants metabolize fatty acids and grow similarly in the presence or absence of sucrose, but β-oxidation mutants have defective growth in the absence of sucrose, making them "sucrose dependent" (Hayashi et al., 1998). The requirement of exogenous sucrose in mutants can be visualized by growing seedlings in the dark or light. To investigate whether fatty acid β-oxidation is compromised in *acx1acx4*, *acx3acx4,* or *acx1acx3acx4,* I grew wild type and *acx* single, double, and triple mutant seeds in the dark in the presence or absence of sucrose. *acx1acx3, acx1acx4* and *acx3acx4* showed normal development on medium lacking exogenous sucrose (Fig. 3-2A). The *acx1acx3acx4* triple mutant displayed a decrease in hypocotyl elongation on medium lacking sucrose, although this defect was minor in contrast to the severe sucrose-dependent phenotype of the *acx1acx2* control (Adham et al., 2005; Pinfield-Wells et al., 2005). The small reduction in *acx1acx3acx4* elongation suggests that this mutant is compromised in fatty acid β-oxidation, but retains a significant amount of activity for germination, seedling establishment, and hypocotyl elongation. For sucrose dependence assays in the light, *acx4*, *acx1acx4*, *acx3acx4,* and *acx1acx3acx4* mutants showed defects in root elongation (Fig. 3-2B), similar to the previously described *acx4* single mutant (Adham et al., 2005). However, a more severe growth defect was observed in *acx1acx2* (Fig. 3-2B). *acx1*, *acx3,* and *acx1acx3* double mutants showed no sucrose dependence in the light (Fig. 3-2B). The sensitivity of all *acx4*-containing mutants is similar, indicating the cause of the short root is not enhanced by additional defects and therefore may be specific to short-chain fatty acid metabolism.

To quantify the fatty acid β-oxidation defect in a more direct manner, GC analysis was conducted on the same lines. Low eicosenoic acid (C20:1) levels are an indicator of seed-storage fatty acid metabolism (Browse et al., 1986), as this lipid moiety accumulates in seeds but is degraded quickly during development (Lemieux et al., 1990). C20:1 levels were measured in wild-type and mutant lines during development. After 6 d of growth, wild-type seedlings have 3.8% eicosenoic acid (vs. total fatty acids), compared to 14.6% remaining in the *acx1acx2* double mutant (Fig. 3-2C). In *acx1acx3acx4*, eicosenoic acid level was at 7%, showing an intermediate phenotype similar to results from hypocotyl elongation assays. This result suggests that the *acx1acx3acx4* mutant does not metabolize eicosenoic acid as efficiently as wild type. However, the defect is not as severe as the *acx1acx2* double mutant, indicating that even with three mutant genes, the plant is able to partially metabolize very-long chain fatty acids.

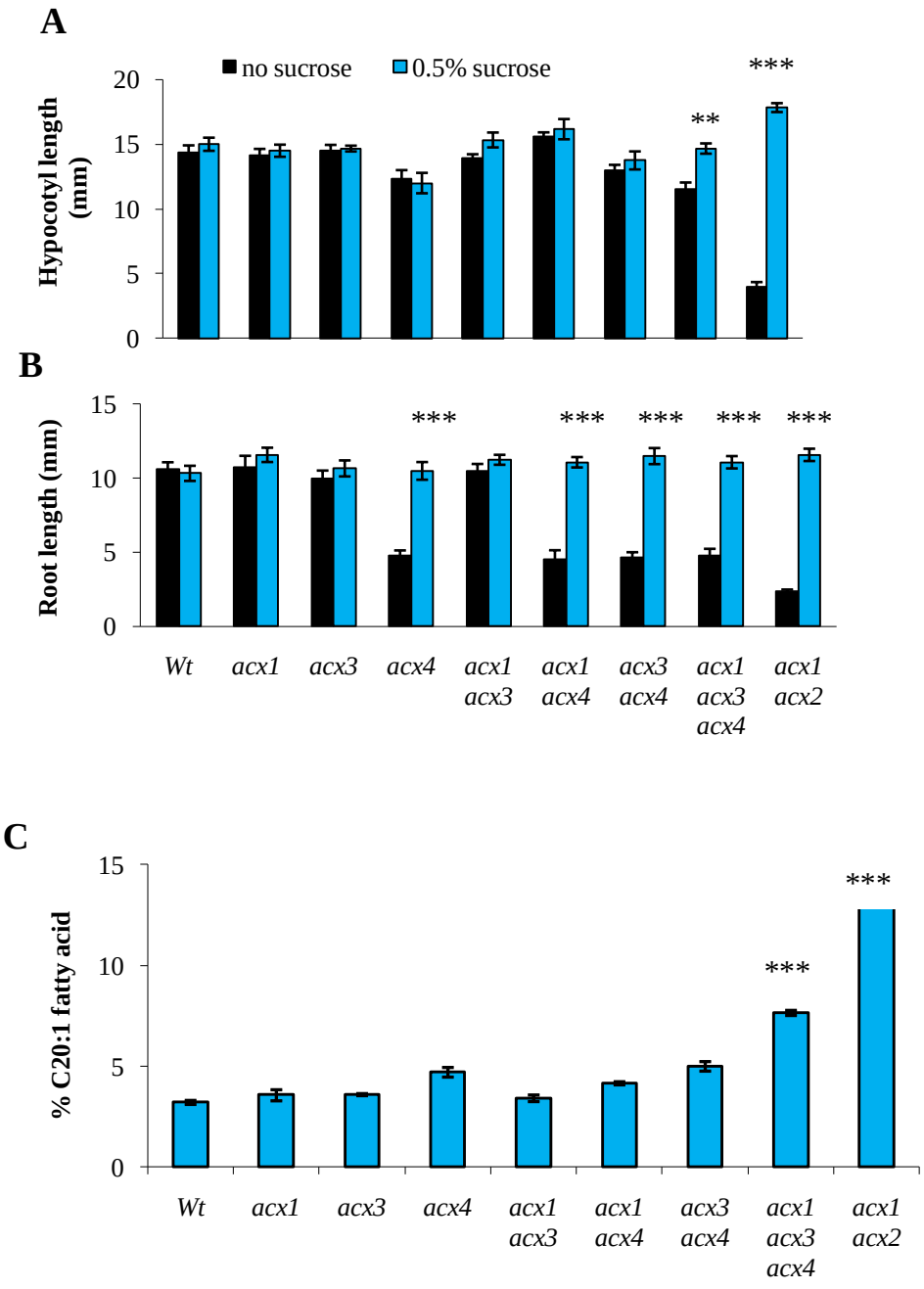


Fig. 3-2. *acx1acx3acx4* triple mutants have minor defects in fatty acid metabolism. (A) Hypocotyl length of wild-type Col-0 (Wt) and *acx* mutant seedlings grown 24 h in light and 5d in dark on medium with or without 0.5% sucrose. Error bars are the standard error of the means($n \geq 12$). (B) Root length of 7 d old seedlings grown under continuous white light on medium with or without 0.5% sucrose. Error bars are the standard error of the means ($n \geq 12$). (C) Percent of C20:1 fatty acids (vs. total fatty acids) in 6 d old light-grown seedlings. Error bars represent the standard error of the means of three biological replicates. *acx1acx2* is shown as a control with a known sucrose-dependent phenotype (Adham et al., 2005;Pinfield-Wells et al., 2005). Values of statistical significance relative to wild-type controls are indicated: ** $P < 0.01$, *** $P < 0.001$.

3.3. ACX activity in *acx1acx3acx4* seedlings

Previous biochemical characterization using *acx* mutant extracts on different fatty acyl-CoA substrates showed reduced activity (Adham et al., 2005). *acx1-2* and *acx1-3* showed a 10-fold reduction in acyl-CoA activity on C16:0, C18:1, and C20:4 acyl-CoA (Adham et al., 2005), suggesting that ACX1 alone may β-oxidize C16-C20 substrates. Medium and short-chain acyl-CoA activity of *acx1-2* and *acx1-3* extracts on C6:0, C12:0, and C14:0 acyl-CoA were similar to that of Wt (Adham et al., 2005). Further, *acx3-4* and *acx3-6* showed only a 2.5 fold reduction in acyl-CoA activity on C12:0 acyl-CoA (Adham et al., 2005), suggesting that other acyl-CoA oxidases may β-oxidize medium-chain substrate and compensate for loss of ACX3 activity. Acyl-CoA activity of *acx3-4* and *acx3-6* extracts on C16:0, C18:0 and short-chain substrates were similar to Wt (Adham et al., 2005). Further, *acx4-1* and *acx4-3* acyl-CoA activity was reduced by three fold on short-chain substrates, such as C4:0 and C6:0, while activity on C12:0, C14:0, C18:1 and C20:4 were similar to Wt (Adham et al., 2005). An *acx4* mutant in Ws background displayed less than 2% acyl-CoA oxidase activity on C6:0 (Rylott et al., 2003). In addition, *acx2-1*, *acx5-1*, *acx6-1* and *acx6-2* displayed Wt acyl-CoA oxidase activity on C6:0, C12:0, C14:0, C18:1 and C20:4 acyl-CoA.

To further investigate the acyl-CoA oxidase defects in double mutants and *acx1-2acx3-6acx4-3*, ACX activity was examined in mutant extracts with various chain length fatty acyl-CoAs. I failed to detect acyl-CoA oxidase activity for *acx1acx2*, *acx1acx3*, *acx1acx4* and *acx1acx3acx4* extracts on C18:1 and C16:0 acyl-CoAs (Fig. 3-3A, B). *acx3acx4* extract showed similar acyl-CoA activity as Wt on both C18:1 and C16:0 acyl-CoAs

(Fig. 3-3A, B). This result is consistent with previous observations (Adham et al., 2005) and suggests that ACX2 cannot compensate for loss of ACX1 on C18:1 and C16:0 acyl-CoA. The results also indicate that ACX1 is the sole acyl-CoA oxidase responsible for the β-oxidation of C18:1 and C16:0.

No detectable acyl-CoA activity was observed for *acx1acx3* and *acx1acx3acx4* on C12:0 acyl-CoA (Fig. 3-3C). However, *acx3acx4* showed residual acyl-CoA activity on C12:0 acyl-CoA (Fig. 3-3C). Acyl-CoA activity was reduced by 20 fold in *acx1acx2* and *acx1acx4* on C12:0 acyl-CoA (Fig. 3-3C). These results suggest that ACX1 and ACX3 have overlapping substrate specificity and while ACX3 is most active on C12:0, ACX1 can partially compensate for loss of ACX3.

β-oxidation of C6:0 acyl-CoA was significantly reduced in *acx1acx4*, *acx3acx4* and *acx1acx3acx4* (Fig. 3-3D), consistent with the previously described *acx4-3* and *acx4-1* single mutants. *acx1acx2* and *acx1acx3* showed similar activity to Wt on C6:0 acyl-CoA (Fig. 3-3D). One previous study showed that a Ws *acx4* mutant had less than 2% of Wt activity, and suggests that perhaps this residual activity is a consequence of ACX3 activity (Rylott et al., 2003). However, in Col-0 *acx3acx4* and *acx1acx3acx4*, there is still residual short chain acyl-CoA activity (Fig. 3-3D).

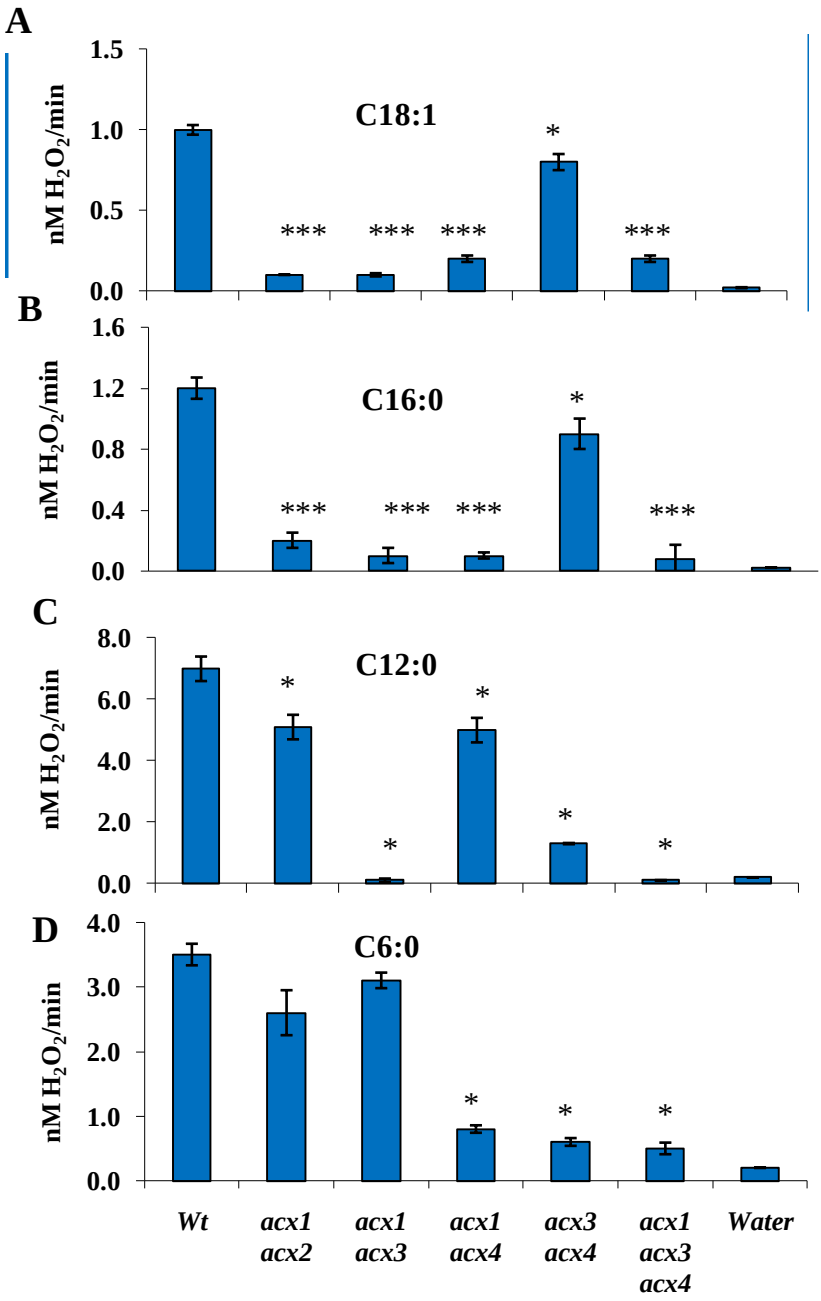


Figure 3-3. Acyl-CoA oxidase activity of *acx* mutants with fatty acid substrates
Extracts from 3 d old light grown Col-0, *acx1acx2*, *acx1acx3*, *acx1acx4*, *acx3acx4* and *acx1acx3acx4* seedlings were tested for acyl-CoA oxidase activity with the following fatty acid substrates (A) oleoyl-CoA (C18:1), (B) palmitoyl-CoA (C16:0) (C) lauroyl-CoA (C12:0), and (D) n-hexanoyl-CoA (C6:0). Water was used as a control to account for background readings. Data are shown in nM H_2O_2 produced per minute. Error bars = standard deviations of rates of three independent experiments. Values of statistical significance relative to wild-type controls are indicated: * P <0.05, ** P < 0.01, *** P < 0.001.

3.4. *acx1acx3acx4* adult phenotype

To investigate whether plant growth and development is compromised in *acx1acx3acx4* mutants, rosette diameter, date of bolting, number of stems and plant height were recorded for Wt, *acx1*, *acx3*, *acx4* single and double mutants and *acx1acx3acx4*. The double and triple *acx* mutants showed decreased plant growth (Fig. 3-4), with the strongest phenotypes seen in *acx1acx3acx4*. However, these defects were minor, and overall plant development and architecture were normal (Fig. 3-4). This data suggests that overall growth and development is not disrupted in the *acx1acx3acx4* mutant.

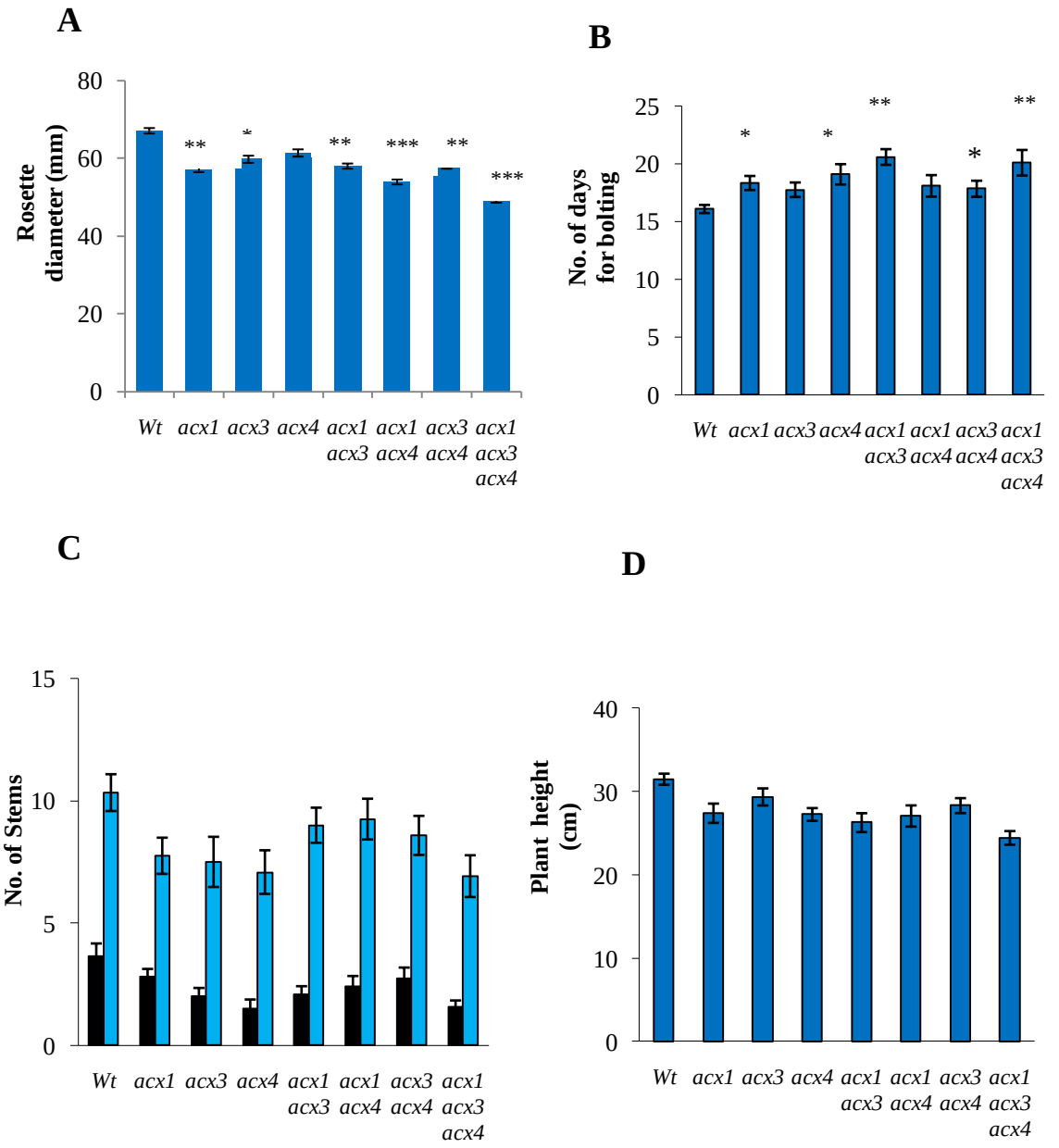


Fig. 3-4. The *acx* triple mutant has normal growth and development.
Col-0 (Wt), *acx1*, *acx3*, *acx4*, *acx1acx3*, *acx1acx4*, *acx3acx4*, and *acx1acx3acx4* plants were grown under continuous white light at 22°C with normal watering two times per week. Plants were measured for (A) rosette diameter at 38 d, (B) the number of days until plants started reproductive growth after transfer to soil, (C) the number of stems produced at 34 and 38 d, and (D) plant height at 38 d. Error bars represent the standard error of the means ($n \geq 18$). Values of statistical significance relative to wild-type controls are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.5. Responses to JA, propionate, and isobutyrate

JA biosynthesis includes three rounds of peroxisomal β-oxidation, requiring ACX enzyme activity (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005; Schilmiller et al., 2007), as discussed in 1.2.c. *acx1acx5* mutants are infertile and show little to no silique elongation, implicating ACX1 and ACX5 in JA biosynthesis (Schilmiller et al., 2007). To determine if ACX3 and ACX4 also play a role in JA synthesis, I examined the triple mutant for JA-response defects. *acx1acx3acx4* plants did not show any fertility defects and silique lengths were similar to wild type. Further, *acx1acx3acx4* seedlings displayed similar root elongation to wild type following application of exogenous JA (Fig. 3-5A). These results suggest that JA synthesis and perception are not appreciably altered in the *acx1acx3acx4* mutant.

CHY1 is a peroxisomal β-hydroxybutyryl-CoA hydrolase (Zolman et al., 2001). *chy1* mutants exhibit enhanced sensitivity to root elongation inhibition on isobutyrate and propionate, which are metabolized in peroxisomes (Lucas et al., 2007). Recently, *chy1* mutants were shown to play a role in cold stress tolerance in Arabidopsis and starvation induced by darkness. This observation showed the involvement of peroxisomes in stress related responses in plants (Donga et al., 2009). *chy1* phenotypes are attributed to an accumulation of toxic catabolic intermediates (Zolman et al., 2001; Lange et al., 2004; Lucas et al., 2007). Therefore, to determine whether the combined loss of *ACX1*, *ACX3*, and *ACX4* affects propionate and isobutyrate processing, Col-0, *chy1-3*, and *acx* mutant seeds were assayed on exogenous propionate and isobutyrate. The *chy1* mutant showed strong inhibition by propionate and isobutyrate (Fig. 3-5B, C). Root elongation of *acx*

mutants all showed a similar degree of weak sensitivity to propionate, except for *acx4* (Fig. 3-5B). The mutants also showed sensitivity to isobutyrate application, but not to the same level as the *chy1* control (Fig. 3-5C). These results suggest that ACX1, ACX3, and ACX4 may play partial or overlapping roles in propionate and isobutyrate metabolism, but this defect is not as strong as *chy1*.

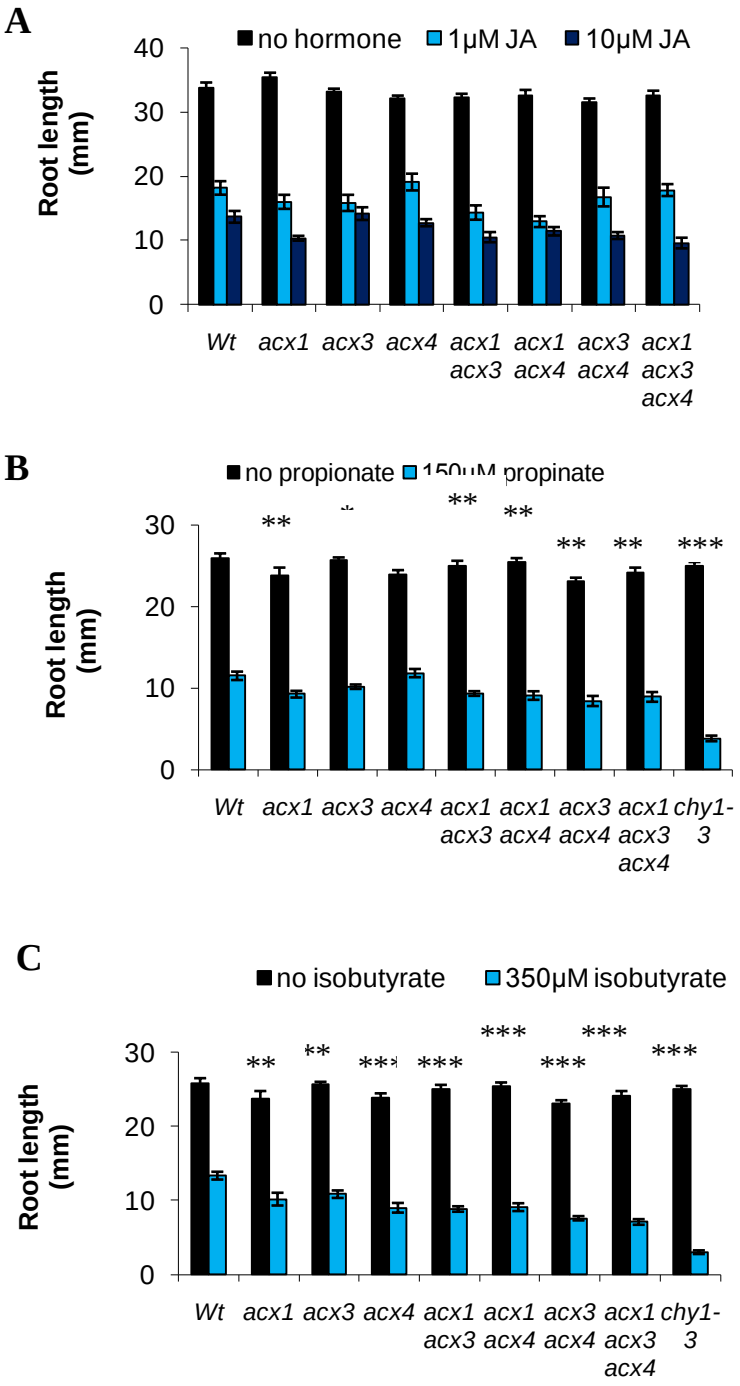
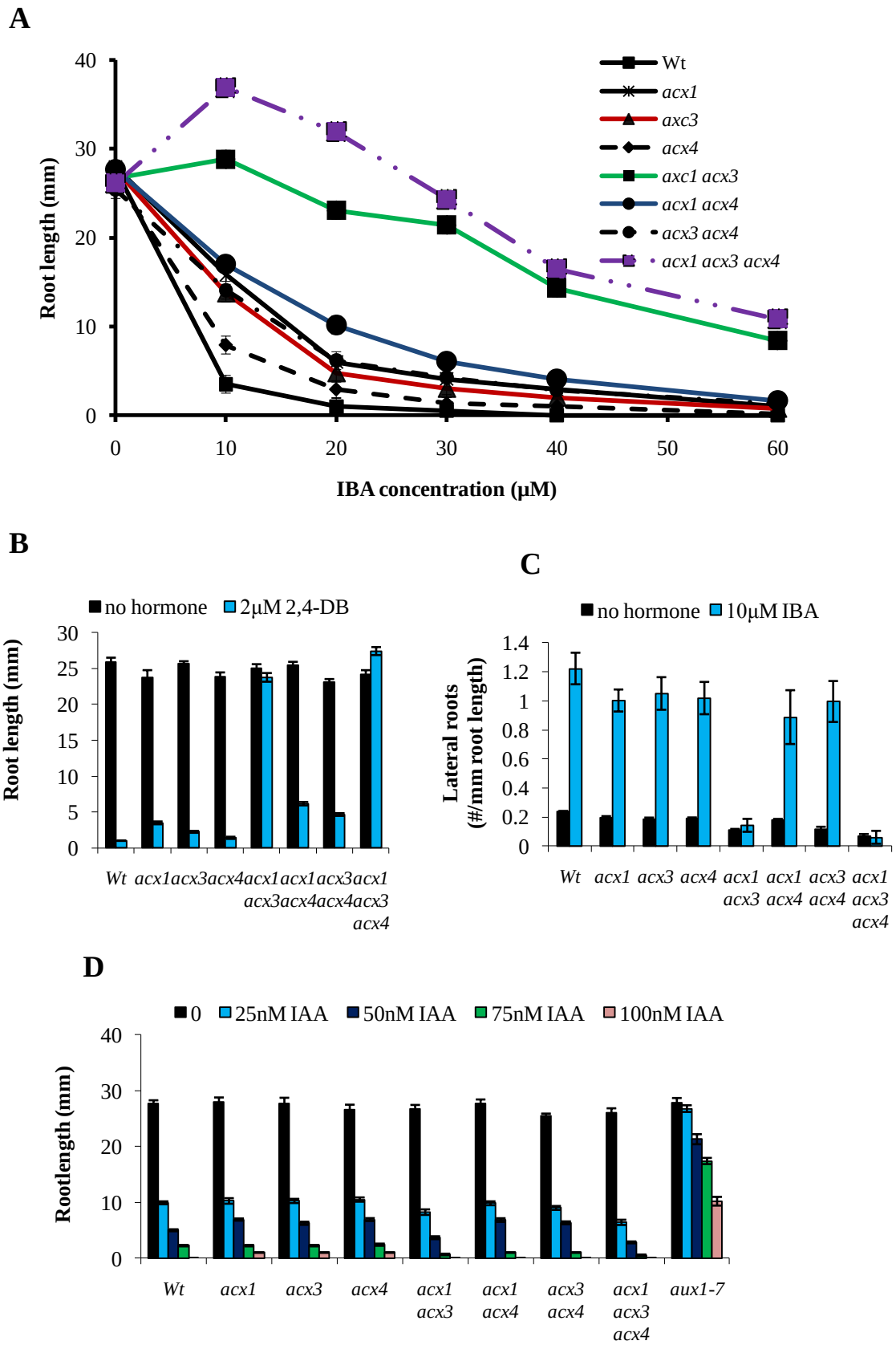


Fig. 3-5. *acx* mutants responses differ on propionate and isobutyrate.
Root lengths of 8 d old Col-0 (Wt) and mutant seedlings grown under continuous white light on 0.5% sucrose medium with (A) 1 µM and 10 µM JA, (B) 150 µM propionate or (C) 350 µM isobutyrate. *chy1-3* was used as a control in the propionate and isobutyrate experiments to show a sensitive phenotype (Lucas et al., 2007). Error bars represent the standard error of the means ($n \geq 12$). Values of statistical significance relative to wild-type controls are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.6. Responses to auxin

As discussed in section 1.2.b., conversion of IBA to IAA is a peroxisomal process and mutants that disrupt this process exhibit an IBA-resistant phenotype in root elongation or lateral root stimulation assays (Zolman et al., 2000). To determine whether *acx1acx3acx4* is defective in IBA to IAA conversion, wild type and *acx* mutants were assayed on inhibitory concentrations of IBA or the IBA analog 2,4-DB. All of the single mutant and higher-order mutants displayed decreased sensitivity to the inhibitory effects of IBA and 2,4-DB on root elongation compared to wild type (Fig. 3-6A, B). However, the *acx1acx3* and *acx1acx3acx4* seedlings were more IBA and 2,4-DB resistant than the other combinations (Fig. 3-6A, B). Although root elongation is greatly enhanced in *acx1acx3* and *acx1acx3acx4*, the roots do show partial sensitivity to IBA at very high concentrations. Application of IBA to wild-type plants stimulates lateral rooting. Mutants defective in IBA responses in root elongation assays show differential responses in lateral root assays: a subset of mutants respond normally to lateral root initiation, whereas others are resistant to the stimulatory effects (Zolman et al., 2000). The single *acx* mutants, *acx1acx4*, and *acx3acx4* have normal lateral root formation (Fig. 3-6C). Decreased lateral rooting is seen in *acx1acx3* but is most severe in the *acx1acx3acx4* mutant (Fig. 3-6C). To determine if the alteration in root responses was specific to IBA and 2,4-DB, I assayed root elongation responses on inhibitory concentrations of IAA in wild type and *acx* mutant lines. *acx1acx3acx4* displayed sensitivity to IAA similar to wild type (Fig. 3-6D). Similar results were seen using the synthetic auxins, 2,4-D and NAA (Fig. 3-6E, F). These results suggest that *acx1acx3acx4* is not disrupting IAA transport, signaling or metabolism, but is specifically compromised in IBA (and 2,4-DB) β-oxidation processes.


A
Root length (mm)
IBA concentration (µM)
Wt
acx1
axc3
acx4
axc1 acx3
acx1 acx4
acx3 acx4
acx1 acx3 acx4
B
no hormone
2µM 2,4-DB
Root length (mm)
Wt
acx1
acx3
acx4
acx1 acx3
acx1 acx4
acx3 acx4
acx1 acx3 acx4
C
no hormone
10µM IBA
Lateral roots (#/mm root length)
D
0
25nM IAA
50nM IAA
75nM IAA
100nM IAA
Rootlength (mm)
aux1-7

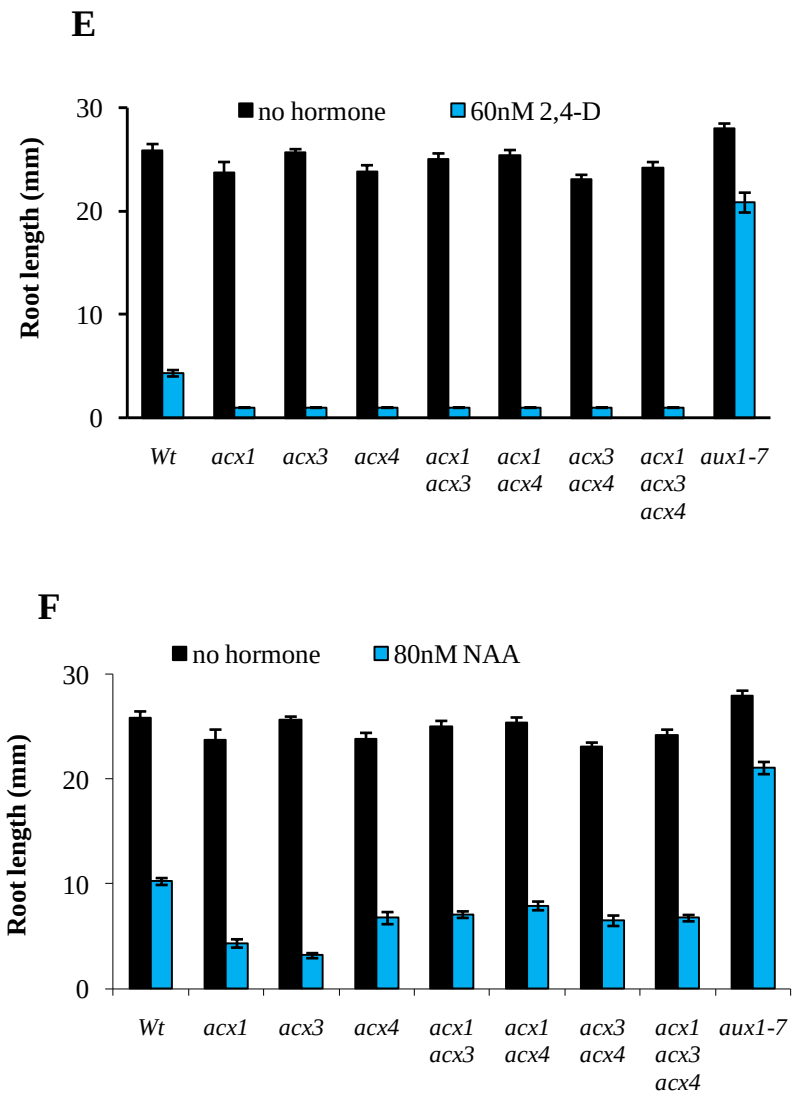


Fig. 3-6. *acx3acx4* and *acx1acx3acx4* are defective in IBA and 2,4-DB responsiveness.

(A-B) Root lengths of 8 d old Col-0 (Wt) and *acx* double and triple mutant seedlings grown under continuous yellow light on medium supplemented with the indicated concentration of (A) IBA or (B) 2 µM 2,4-DB (D) IAA, (E) 2,4-D or (F) NAA. *aux1-7* is an auxin resistant mutant (Pickett et al., 1990) shown as a control. Error bars represent the standard error of the means ($n \geq 12$). (C) Lateral root initiation of seedlings grown on 0.5% sucrose medium for 5 d, followed by transfer to 10 µM IBA for another 5 days. Data points are the number of lateral roots per mm root length. Error bars represent the standard error of the means ($n \geq 12$).

3.7. *acx* mutants in Col-0 and Ws backgrounds

The data reveal that the *acx* double and triple mutants in the Col-0 background have several phenotypes indicating disruption of peroxisomal metabolism. Interestingly, the *acx3acx4* double mutants have different phenotypes in the Col-0 and Ws accessions: the Col-0 double mutant has defects in fatty acid metabolism (Fig. 3-3) and IBA responses (Fig. 3-6) but relatively normal adult growth and development (Fig. 3-4), whereas the Ws double mutant is embryo lethal (Rylott et al., 2003). To determine if this defect simply results from differences in allele strength, I compared acyl-CoA oxidase activity in the Col-0 mutant alleles with the Ws versions of *acx1-1* (Pinfield-Wells et al., 2005), *acx3*-1 (Eastmond et al., 2000), and $acx4_W$ (Rylott et al., 2003). Compared to the respective wild-type parents, each of the mutants showed a similar response pattern and degree of activity loss (Fig. 3-7A-C). I also compared the IBA-response defects in the mutant alleles compared to the respective wild types. Similarly, each of the mutants showed a comparable degree of inhibition (Fig. 3-7D). In addition to IBA response, I compared the Col-0 and Ws mutants in other assays indicative of peroxisomal function. The *acx4* mutant alleles showed a differential response following propionate application; whereas the Col-0 allele showed only a slight alteration from wild type (86% elongation compared to the no hormone control), the Ws mutant showed stronger growth inhibition (54% elongation; Fig. 3-7E). This hypersensitivity could indicate that the Ws mutant leads to an accumulation of a toxic intermediate. Alternatively, *acx1* and *acx3* mutant alleles in both backgrounds showed similar responses (Fig. 3-7E). None of the single mutants showed a sucrose dependent phenotype in the dark (Fig. 3-7F).

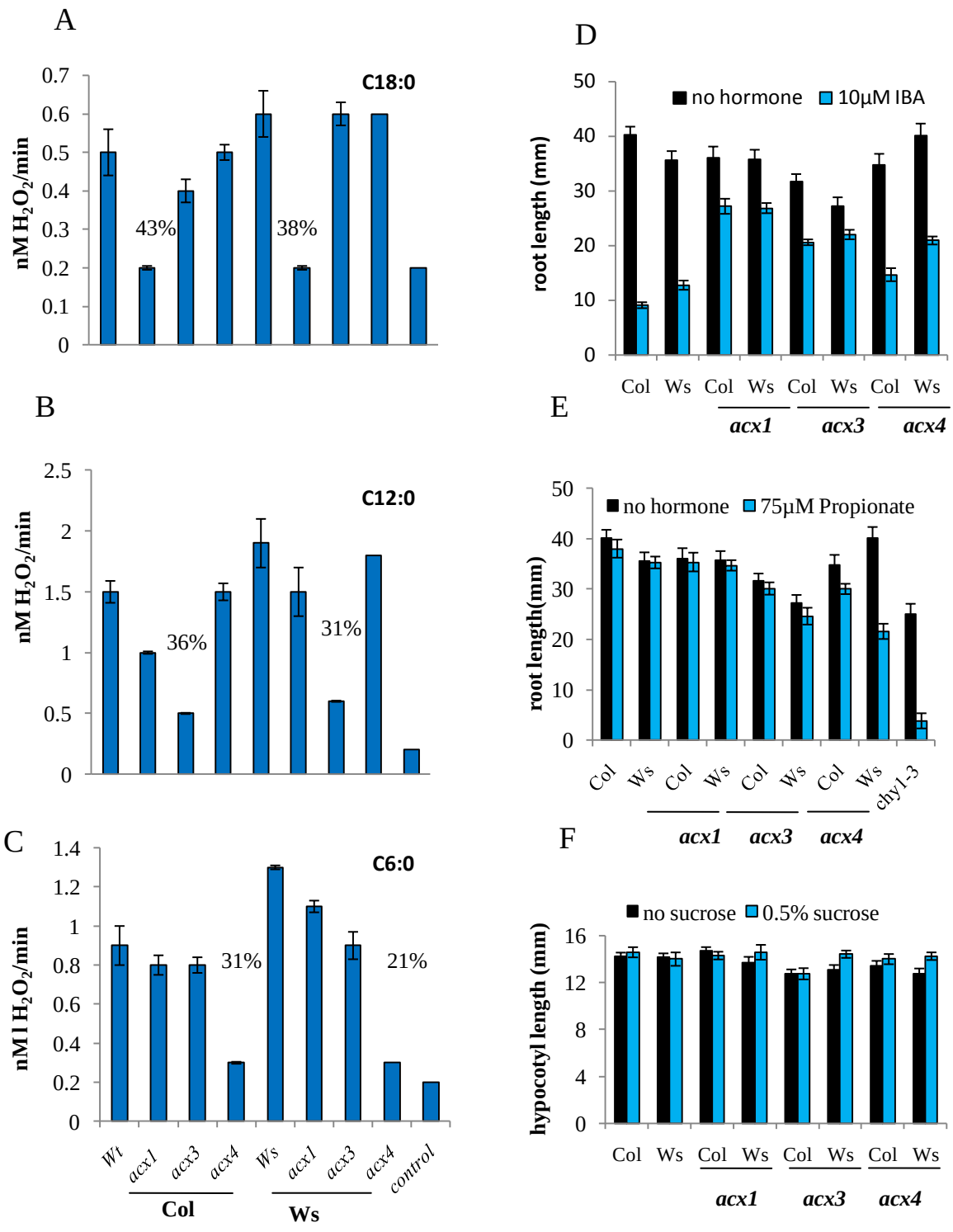

A
C18:0
nM H2O2/min
43%
38%
B
C12:0
nM H2O2/min
36%
31%
C
C6:0
nM H2O2/min
31%
21%
Wt
acx1
acx3
acx4
Ws
acx1
acx3
acx4
control
Col
Ws
D
no hormone
10µM IBA
root length (mm)
Col
Ws
Col
Ws
Col
Ws
Col
Ws
acx1
acx3
acx4
E
no hormone
75µM Propionate
root length(mm)
Col
Ws
Col
Ws
Col
Ws
Col
Ws
chy1-3
acx1
acx3
acx4
F
no sucrose
0.5% sucrose
hypocotyl length (mm)
Col
Ws
Col
Ws
Col
Ws
Col
Ws
acx1
acx3
acx4

Fig. 3-7. *acx* single mutant from Col-0 and Ws backgrounds.
(A-C) Extracts from 4 d old light grown Col-0, Ws, and *acx* single mutant alleles from the Col-0 and Ws backgrounds were tested for acyl-CoA oxidase activity with (A) oleoyl-CoA (C18:1), (B) lauroyl-CoA (C12:0), or (C) n-hexanoyl-CoA (C6:0), as described in Fig. 3. Error bars represent the standard error of the rates from three independent experiments, except the *acx4* (Ws) sample which shows the average of two independent experiments. For easy comparison between Col-0 and Ws alleles, percent values are listed above the standard substrate for each mutant. (D-E) Root lengths of 12 d old Col-0 and Ws wild-type seedlings and *acx* mutant alleles grown under continuous yellow light on medium supplemented with (D) IBA or (E) propionate. (F) Hypocotyl length of Wt and mutants grown in light for 1 day and dark for 6 days. Black bars indicate control growth and grey bars represent experimental conditions. Error bars represent the standard error of the means ($n \geq 12$).

3.8. ACX activity in Col-0 vs. Ws

Because *acx* mutants in the Col-0 and Ws accessions showed different phenotypes, I wanted to investigate ACX expression and activity in wild-type Arabidopsis plants. Differential *ACX* gene expression, protein accumulation, or protein activity in Arabidopsis accessions could account for the differences in mutant phenotypes. First, I examined gene expression levels for the five *ACX* genes in the Col-0 and Ws backgrounds. Four of the genes showed similar expression levels, but *ACX1* consistently was lower in the Ws background (Fig. 3-1D, E). To examine whether acyl-CoA oxidase activity differs in Col-0 and Ws accessions, I performed ACX activity assays with Col-0 and Ws at different developmental stages. Because the *acx3acx4* mutant in the Ws accession is embryo lethal (Rylott et al., 2003), I tested activity in embryos. Both Col-0 and Ws embryos showed measurable oxidase activity on C6:0 and C12:0 (Fig. 3-8A and B), albeit at reduced rates compared to older seedlings. Acyl-CoA oxidase activity was tested in Ws and Col-0 at 46 hr (radicle emergence; Fig. 3-8C-E) and 60 hr (cotyledon emergence; Fig. 3-8F-H) after plating. Although *ACX1* expression levels were reduced in Ws, Ws samples consistently showed similar or higher activity compared to Col-0 extracts (Fig. 3-7A-C,3-8). Finally, I investigated ACX activity under different conditions. I tested activity of 3 d old seeds, grown in the dark in the presence or absence of sucrose. As expected, overall acyl-CoA oxidase activity in Ws was higher in seedlings compared to Col-0 seedlings (Fig. 3-9A). For most of the conditions tested, oxidase activity was greater in seedlings grown in the absence of sucrose, although C18:1 oxidation was not strongly affected by sucrose concentrations (Fig. 3-9). For C6:0 and C12:0, Col-0 seedlings grown without sucrose had similar acyl-CoA activity to Ws

seedlings grown on sucrose (Fig. 3-9). These results demonstrate that seedlings grown in the absence of sucrose have higher acyl-CoA oxidase activity than in the presence of sucrose. These results support the idea that both Col-0 and Ws seedlings can alter β-oxidation activity based on changing environmental conditions.

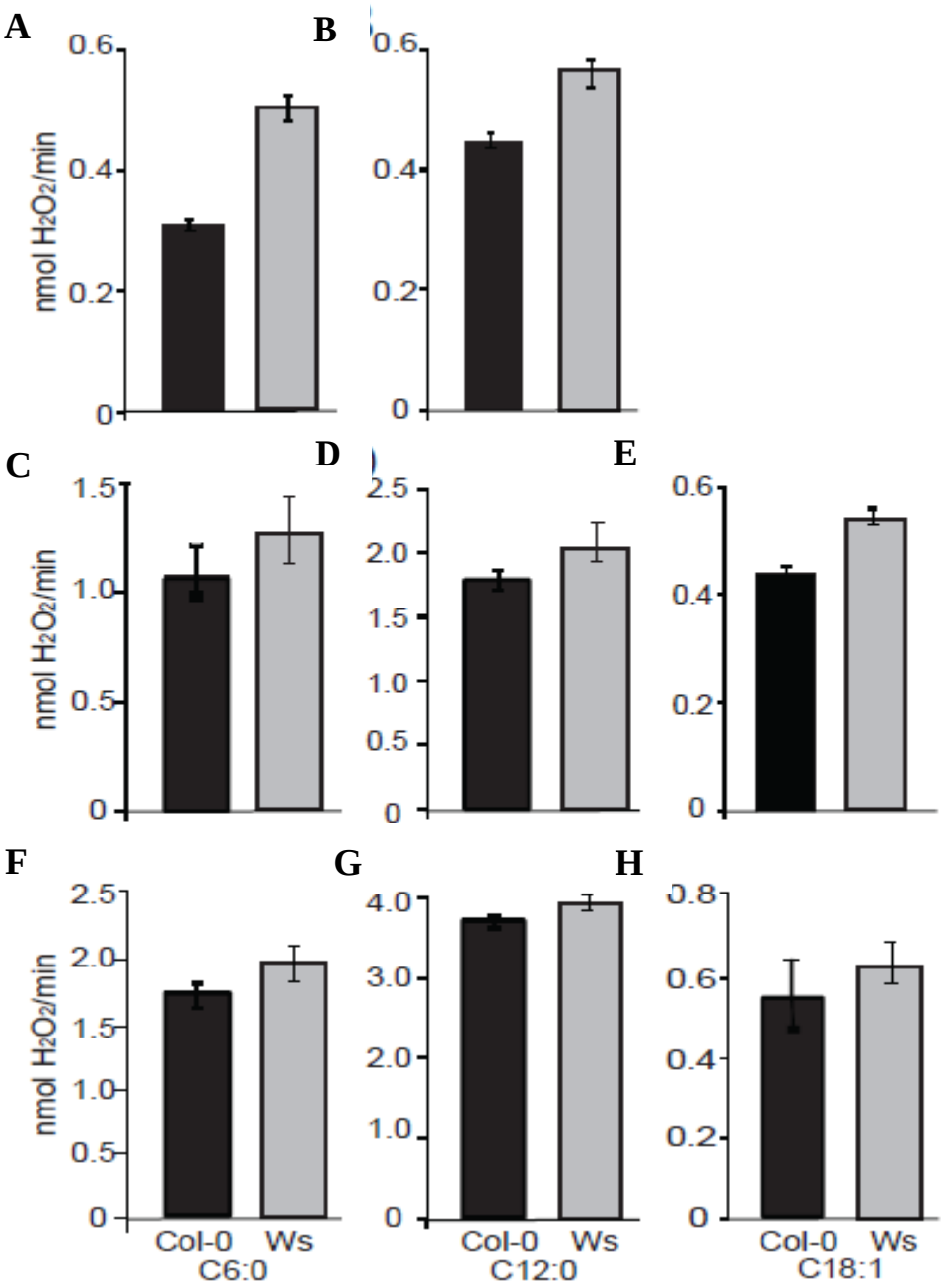


Fig. 3-8. Acyl-CoA oxidase activity in Col-0 and Ws wild-type samples.
Extracts from embryos (A, B), 46 hr (C, D, E) and 60 hr old (F, G, H) light grown Col-0 (black bars) and Ws (gray bars) seedlings were tested for ACX activity with (A, C, F) n-hexanoyl-CoA (C6:0), (B, D, G) lauroyl-CoA (C12:0), and (E, H) oleoyl-CoA (C18:1) substrates. Error bars represent the standard error of rates from three independent experiments.

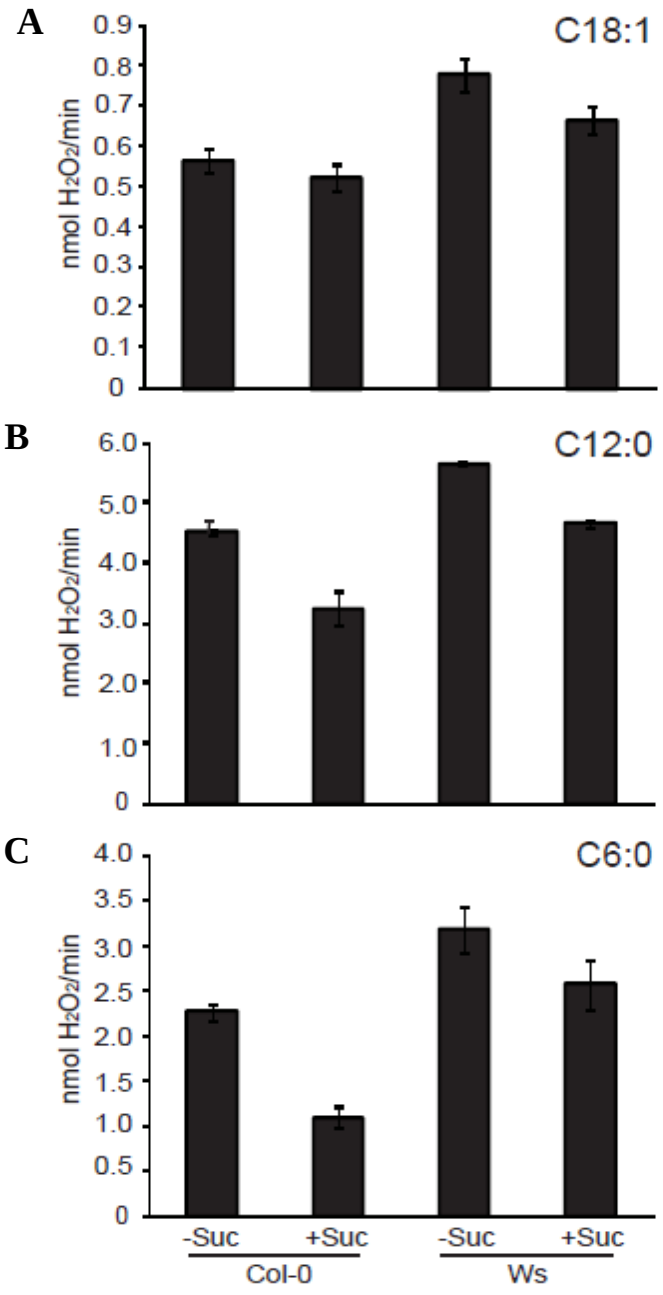


Fig. 3-9. Acyl-CoA oxidase activity under different conditions.
Extracts from 3 d old Col-0 and Ws seedlings grown in darkness on medium with or without 0.5% sucrose were tested for acyl-CoA oxidase activity with (A) oleoyl-CoA (C18:1), (B) lauroyl-CoA (C12:0), and (C) n-hexanoyl-CoA (C6:0) substrates. Error bars represent the standard error of rates from three independent experiments.

3.9. Mixed double mutants

To determine whether the lethality of the *acx3acx4* double mutant in the Ws ecotype (Rylott et al., 2003) is only specific to the Ws ecotype I crossed various single *acx3* and *acx4* alleles in Col and Ws accessions. The *acx3-2* Ws allele was crossed to *acx4-1* and *acx4-3* Col-0 alleles and a Ws *acx4* mutant allele was crossed to the *acx3-6* Col-0 allele. The *acx3-2$_W$acx4-3* and the *acx3-6acx4$_W$* double mutant are in the F_2 generation and are being genotyped to isolate homozygous combinations for further phenotypic assays. In this section, I describe the phenotypes of the *acx3-2$_W$acx4-1* double mutant.

Two homozygous plants were isolated for the *acx3-2$_W$acx4-1* double mutant (L14, L59), these plants were screened to determine whether the *ACX1* and *ACX2* genes were from the Ws or Col-0 ecotype, since *ACX1* is more highly expressed in Col-0 than in Ws and *ACX2* is up-regulated in the *acx1acx3acx4* triple mutant. The *ACX1* in *acx3-2$_W$acx4-1* (L14) was determined to be from the Ws ecotype while *ACX2* was from Col-0 ecotype. The *ACX1* in *acx3-2$_W$acx4-1* (L59) was from Col-0 ecotype and *ACX2* was from Ws ecotype. *acx3-2$_W$acx4-1* (L14) and (L59) were more IBA resistant than either parent (Fig. 10A) indicating that IBA β-oxidation is more defective in this double mutant than the respective single mutants. *acx3-2$_W$acx4-1* (L14) and (L59) were severely sucrose dependent while the single mutants were not sucrose dependent (Fig. 3-10B). Hypocotyl elongation was severely inhibited on media lacking sucrose and this phenotype was partially rescued on media supplemented with 0.5% sucrose. Further *acx3-2$_W$acx4-1* (L14) and (L59) showed delayed germination (Table 3-1). These results taken together indicate that fatty acid β-oxidation is severely compromised in the mixed *acx3-2$_W$acx4-1*

double mutant. Importantly, although this mutant has a strong fatty acid β-oxidation phenotype the mutant is viable. The *acx3acx4* double mutant in Col-0 is not sucrose dependent. Thus, the dependence of the *acx3-2*$_W$*acx4-1* mixed double mutant on sucrose indicates that ACX3 in Ws is critical for fatty acid β-oxidation.

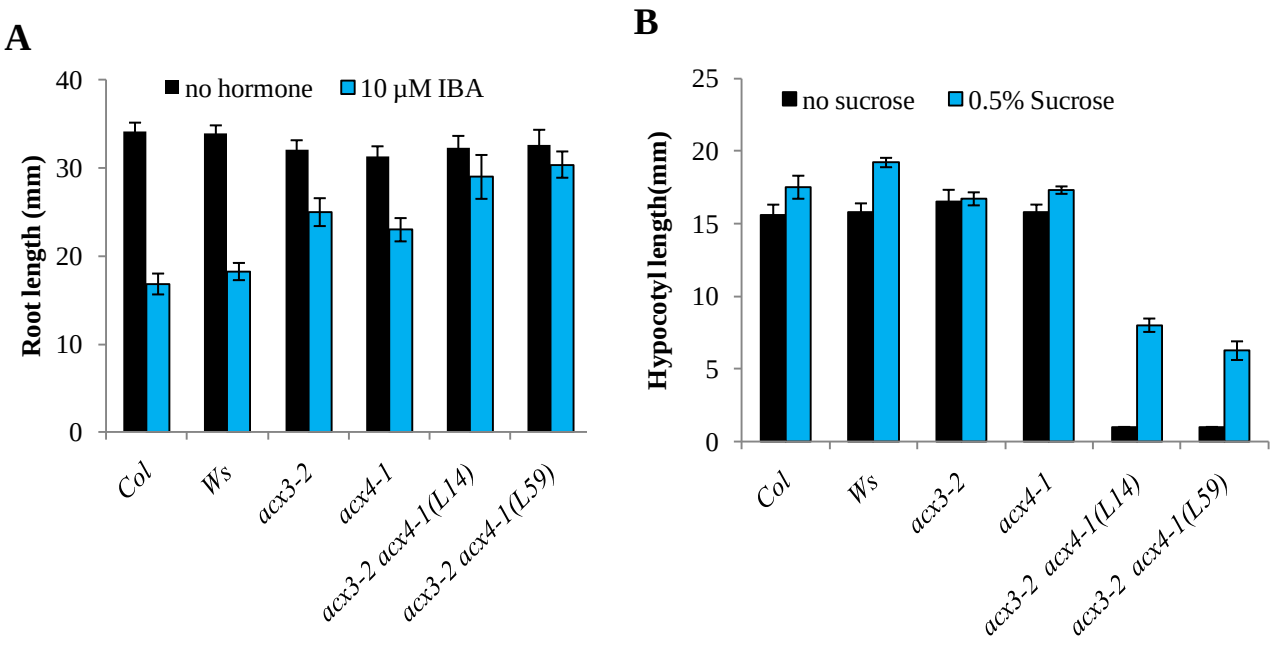


Fig. 3-10. *acx* mixed double mutants from Col-0 and Ws backgrounds.
(A)Root lengths of 12 d old Col-0 and Ws wild-type seedlings and *acx* single and mixed double mutant alleles grown under continuous yellow light on medium supplemented with IBA. (B) Hypocotyl length of Wt and mutants grown in light for 1 day and dark for 6 days. Black bars indicate control growth and grey bars represent experimental conditions. Error bars represent the standard error of the means ($n \geq 12$).

Table. 3-1. Germination rate of 100 seeds.

Plants	Day1	Days2	Day3	Day4	Day5
Col-0	0	57	100		
acx3-2	0	45	100		
acx4-1	0	50	100		
acx3-2acx4-1 (L14)	0	2	10	40	100
acx3-2acx4-1 (L59)	0	0	5	37	100

3.10. Discussion

One previous study showed a striking *acx* double mutant, *acx3acx4* from the Ws background; this double mutant is embryo lethal, indicating a severe disruption in metabolism or the buildup of a toxic intermediate for which the plant is unable to compensate (Rylott et al., 2003). In this chapter, I further investigated the roles of the ACX1, ACX3 and ACX4 enzymes in plant growth and development. I isolated and characterized an *acx3acx4* and *acx1acx3acx4* triple mutant in an Arabidopsis Col-0 accession. I found that generation of an *acx3acx4* double mutant in the Col-0 background was feasible and that the mutant had normal growth. Several possible reasons might explain this finding. First, one or both of the *acx* mutants in the Ws background could be stronger alleles than the corresponding mutants in the Col-0 accession. However, examination of the mutant alleles in a side-by-side assay revealed that the defects led to similar degrees of enzyme activity loss (Fig. 3-7A-C) and IBA resistance (Fig. 3-7D), indicating that the mutant strength, at least in these assays, is similar.

A second possibility is that the difference in the *acx3acx4* mutant phenotypes is based on differences in the accessions themselves. For instance, the Col-0 *acx4* mutant has altered growth in the absence of sucrose in the light (Adham et al., 2005), whereas the Ws mutant does not (Rylott et al., 2003). This defect in Col-0 *acx4* is maintained in the Col-0 *acx1acx4* and a*cx3acx4* double mutants (Fig. 3-2B). Additionally, I found that responses to propionate in the Ws and Col-0 *acx4* single mutants were not equal; the Ws allele is more sensitive to propionate application than the Col-0 version (Fig. 3-7B). Either a) the ability of the other ACX enzymes (or other peroxisomal proteins) to compensate for the

disrupted gene is not equal between the accessions, based on differences in protein accumulation or activity; or b) the β-oxidation of substrates is differentially important in the different accessions, leading to less of a necessary product or more of a toxic intermediate. Certainly, these types of differences between Arabidopsis accessions are not unique; differential effects between accessions previously have been reported in numerous genes affecting various aspects of plant growth and development (Shindo, 2007). These differences in function can be due to coding sequence changes, gene expression levels, protein activity, or enzyme regulation.

To further explore the activity of the ACX enzymes in each Arabidopsis background, I examined gene expression and protein activity in Col-0 and Ws. The Col-0 and Ws ACX protein sequences are highly similar, with the few alterations being only minor amino acid conversions or changes in non-conserved residues. I examined *ACX* expression levels by Northern blot analysis in Col-0 and Ws seedlings and found that each gene was expressed similarly in the two backgrounds except for *ACX1* (Fig. 3-1). *ACX1* consistently showed lower expression in the Ws background than in Col-0. Because Ws plants are able to metabolize long-chain substrates (Fig. 3-8), the decreased expression of *ACX1* may not normally be affecting plant growth and development. However, it is possible that ACX3 and ACX4 normally compensate for ACX1, perhaps explaining why the *acx3acx4* mutant in Ws is embryo lethal while the Col-0 version is normal. Generation of double mutant combining the Col-0 and Ws alleles, a*cx3-2*$_W$*acx4-1* was viable with a strong dependence on sucrose indicating a block in fatty acid β-oxidation. The hypocotyl elongation and germination defects seen in *acx3-2*$_W$*acx4-1* but not in Col-

0 *acx3acx4* suggest that ACX3 is playing a greater role in fatty acid β-oxidation in Ws than Col-0.

In addition to changes in gene expression, I compared the acyl-CoA oxidase activity in wild-type Col-0 and Ws during development and under different conditions. Col-0 and Ws showed similar activity on all substrates tested at 46 hr and 60 hr, whereas Ws showed higher activity during embryogenesis (Fig. 3-8). Both Col-0 and Ws seedlings showed higher activity in the absence of sucrose than when sucrose was included in the growth media (Fig. 3-9), demonstrating how acyl-CoA oxidase activity varies during development and under different conditions.

The most surprising finding was that I could generate an *acx1acx3acx4* triple mutant, and furthermore, that it had no outstanding phenotypes. When I began this project, I expected that disruption of the three most highly-expressed enzymes that metabolized the complete range of fatty acid substrates would have a detrimental impact on plant growth and development, perhaps to the degree of lethality. Again, partial activity in the mutants may explain this apparently normal development. Alternatively, another enzyme with short chain oxidase activity (other than ACX1/ACX3) could compensate for loss of ACX4. Although *acx1acx3acx4* shows a complete block in long- and medium-chain fatty acid β-oxidation (Fig. 3-3), the triple mutant can partially metabolize C20:1 seed-storage fatty acids (Fig. 3-2C). Endogenous short-chain fatty acyl-CoA substrates may be reduced for any acx4 activity, suggesting that the partial activity remaining in these mutants cannot completely explain why the triple mutant is still alive.

There are a few other possibilities that could explain why the triple mutant can grow similarly to wild-type. First, ACX2 and ACX5 may cover for the loss of the three missing enzymes. Although the triple mutant is not as sucrose dependent as the *acx1acx2* double mutant, I see a minor phenotype (Fig. 3-2A and B), indicating that the retention of ACX2 is supporting the ability of the triple mutant to be alive. In addition, *ACX2* is more strongly expressed in the *acx1acx3acx4* triple mutant than wild type (Fig. 3-1C), indicating that the plant may be able to cross-regulate these genes and increase their expression when other ACX family members are absent or disrupted. However, ACX2 has a limited range of substrate specificities (C10:0-C20:0, with highest activity on C18:0 and no activity on substrates below 10 carbons; (Hooks et al., 1999). ACX5 has not been tested *in vitro* for enzymatic activity but only is expressed at a low level (Fig. 3-1). The *acx5* mutant only showed phenotypes when combined with *acx1,* as seen by the enhanced JA-response defects (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005; Schilmiller et al., 2007) and IBA-resistant root elongation (Adham et al., 2005) in the double mutants. Taken together, these results indicate that ACX5 may be redundant to ACX1 and is normally masked by the higher expression of *ACX1*. An alternative theory is that other peroxisomal enzymes or metabolic pathways in other organelles can compensate for the loss of these fatty acid β-oxidation enzymes. Future work, perhaps involving generation of an *acx* quadruple mutant, will further reveal the importance of these enzymes in early plant growth.

The overall goal is to identify the specific enzymes that act in individual peroxisomal processes. Fatty acid β-oxidation is one of the major peroxisomal process in plants and

the ACX proteins play a well-characterized role in this pathway. In this study, I showed that in the absence of sucrose in the dark, the *acx1acx4* and *acx3acx4* double mutants establish as well as wild-type plants, whereas the *acx1acx3acx4* triple mutant had shorter hypocotyls than wild-type and *acx1acx2* has a dramatic reduction in growth (Fig. 3-2A). Consistent with the previously described sucrose dependent phenotype of *acx4* in the light (Adham et al., 2005) *acx1acx4*, *acx3acx4* and *acx1acx3acx4* also had short roots in the light in the absence of sucrose (Fig. 3-2B). GC analysis of remaining C20:1 in 6 d old seedlings revealed that *acx1acx3acx4* β-oxidized C20:1 fatty acids less effectively than wild-type, but better than *acx1acx2* (Fig. 3-2C). *acx1acx2*, *acx1acx3*, *acx1acx4* and *acx1acx3acx4* mutants completely lacked oxidase activity on long-chain fatty acid substrates (Fig. 3-3A), indicating that ACX1 is the primary enzyme involved in the β-oxidation of long-chain fatty acid substrates. Compared to wild-type, *acx1acx2* and *acx1acx4* mutants showed a slight reduction in oxidase activity on C12:0 fatty acid substrates (Fig. 3-3B). *acx1acx3* and *acx1acx3acx4* mutants lacked detectable activity on C12:0 (Fig. 3-3B), whereas *acx3acx4* mutants retained some activity (Fig. 3-3C). These results suggest that ACX1 can partially compensate for loss of ACX3. ACX4 plays the primary role in short-chain β-oxidation, with only minor contributions from ACX1 and ACX3.

JA biosynthesis also involves peroxisomal β-oxidation. Previous results suggest that ACX1 is responsible for the majority of JA synthesis in Arabidopsis, consistent with its dramatic increase in expression in response to wounding (Fig. 3-1C). ACX5 appears to play a redundant role in JA biosynthesis, which is only observed in the absence of ACX1

(Schilmiller et al., 2007). *acx1acx3acx4* has a normal response in JA bioassays (Fig. 3-5A and B). Because an *acx1acx5* double mutant has strong defects in JA responses (Schilmiller et al., 2007), I surmise that ACX5 is able to compensate for loss of *ACX1* in *acx1acx3acx4*, and that ACX3 and ACX4 are not acting in this process.

Finally, IBA is a proto-auxin that is metabolized in peroxisomes to IAA when the plant needs to increase IAA levels (Zolman et al., 2000). *acx* single mutants have IBA-resistant phenotypes in root elongation assays (Adham et al., 2005), but the role of ACX enzymes in oxidizing IBA intermediates has not been clarified. The mutants may be resistant due to loss of enzymatic activity on IBA substrates, or due to an indirect disruption of IBA β-oxidation, perhaps due to limiting cofactor concentrations in the peroxisome (Adham et al., 2005). *acx3acx4* and *acx1acx4* were more IBA resistant than the single mutants (Fig. 3-6A and B). *acx1acx3* and *acx1acx3acx4* demonstrated the greatest enhancement of root elongation on inhibitory concentrations of IBA (Fig. 3-6A and B). *acx1acx3acx4* made fewer lateral roots that any of the single or double mutants tested, both without induction and with IBA stimulation (Fig. 3-6C). The slight increases in phenotype between the *acx1acx3* mutant and the triple mutant correspond to the weak phenotype in *acx4*. This level of resistance, therefore, may reflect the complete block of IBA metabolism by the parallel fatty acid β-oxidation pathway. Alternatively, these results could indicate that, regardless of their apparent substrate specificity, all of the ACX enzymes act on IBA substrates.

CHAPTER 4: Characterization of additional Acyl-CoA oxidase (ACX) triple mutants in Arabidopsis Columbia-0 accession

In this chapter, I present additional *acx* triple mutants in the Col-0 accession to continue to understand the role of ACX enzymes in fatty acid β-oxidation and other peroxisomal β-oxidation processes. ACX1, ACX3 and ACX4 were considered to be the most active in β-oxidation processes, however, the triple showed no significant phenotype. Thus, in this chapter I examined the contributions of ACX2 and ACX5 in peroxisomal β-oxidation processes. To do this I characterized eight *acx* triple mutants. Crosses to generate the double and triple mutants are shown in Table 2-1. Dr. Zolman did the mutant crosses and I genotyped the progenies to isolate homozygous mutants. I further confirmed these mutants by RT-PCR and completed all phenotypic analysis. Below I describe the results for these mutant characterizations.

4.1. Mutant isolation

PCR analysis was used to genotype F_2 progeny to isolate *acx1acx5*, *acx2acx3*, *acx2acx4*, *acx2acx5*, *acx3acx5*, *acx4acx5*, *acx1acx2acx3*, *acx1acx2acx4*, *acx1acx2acx5*, *acx1acx3acx5*, *acx1acx4acx5*, *acx2acx3acx4*, *acx2acx3acx5*,*acx2acx4acx5* and *acx3acx4acx5* mutants. Genomic primers spanning the site of the T-DNA insertions in *ACX1*, *ACX2*, *ACX3*, *ACX4* and *ACX5* amplified the expected products in Wt plants but failed to amplify any product in the various mutant combinations (Fig. 4-1A). Amplification of mutant plants with *ACX1*, *ACX2*, *ACX3*, *ACX4* and *ACX5* genomic

primers 3' to the T-DNA insert and the LB1Salk primer resulted in a product, while no product was amplified in Wt plants (Fig. 4-1A). This result suggests that homozygous *acx1acx2acx3, acx1acx2acx4, acx1acx2acx5, acx1acx3acx5, acx1acx4acx5, acx2acx3acx4, acx2acx3acx5, acx2acx4acx5* and *acx3acx4acx5* triple mutants were successfully isolated. All primers used for genotyping mutants are listed in Table 2-2.

To confirm the homozygosity of the triple mutants, RT-PCR was performed on total RNA isolated from 8 d old seedlings except for *acx2acx4acx5*. Primers spanning the sites of the T-DNA insert in *acx1, acx2, acx3, acx4* and *acx5* failed to amplify a product in the cDNA of the various combinations triple mutant but amplified the expected product for Wt cDNA (Fig. 4-1B).

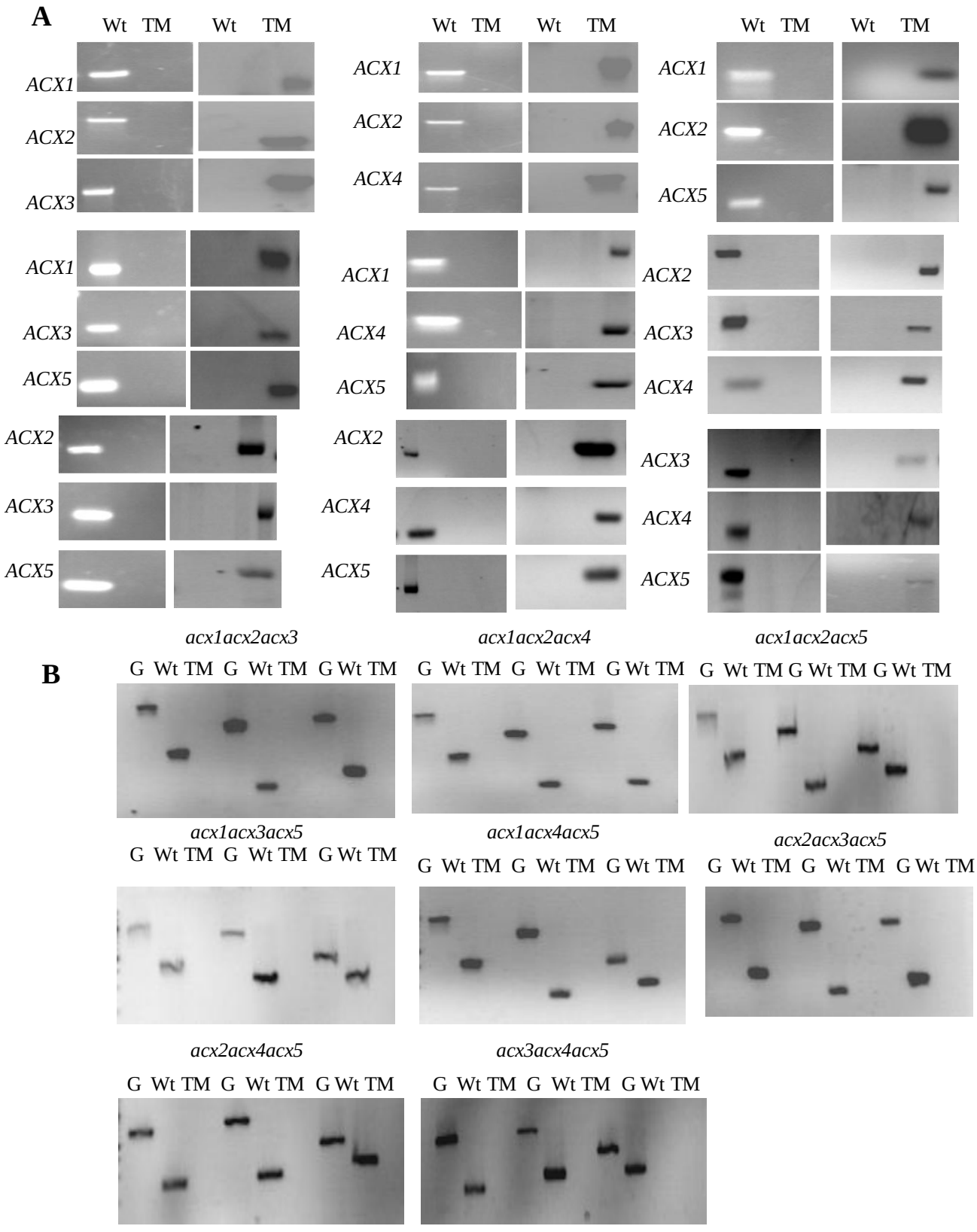

A
Wt TM Wt TM
ACX1
ACX2
ACX3
ACX4
ACX5
acx1acx2acx3
acx1acx2acx4
acx1acx2acx5
B
G Wt TM G Wt TM G Wt TM
acx1acx3acx5
acx1acx4acx5
acx2acx3acx5
acx2acx4acx5
acx3acx4acx5

Figure 4-1. Isolation and confirmation of *acx* triple mutants.
(A) DNA was extracted from *acx* triple mutants and PCR-amplified with *ACX1*, *ACX2*, *ACX 3, ACX4* and *ACX5* gene-specific and T-DNA primers as appropriate for the specific mutant. DNA from Wt Col-0 was used as a control with both set of primers in the PCR reactions.
(B) cDNA was synthesized from RNA isolated from 3 d old Col-0 and *acx* triple mutant (TM) seedlings. Col-0 and mutant cDNAs were amplified with *ACX1*, *ACX2*, *ACX3*, *ACX4* or *ACX5* specific primers spanning the site of T-DNA insertions. Genomic DNA (G) from Wt, Col-0 was amplified with all primer sets to show success of cDNA synthesis.

4.2. Fatty acid β-oxidation in additional *acx* triple mutants

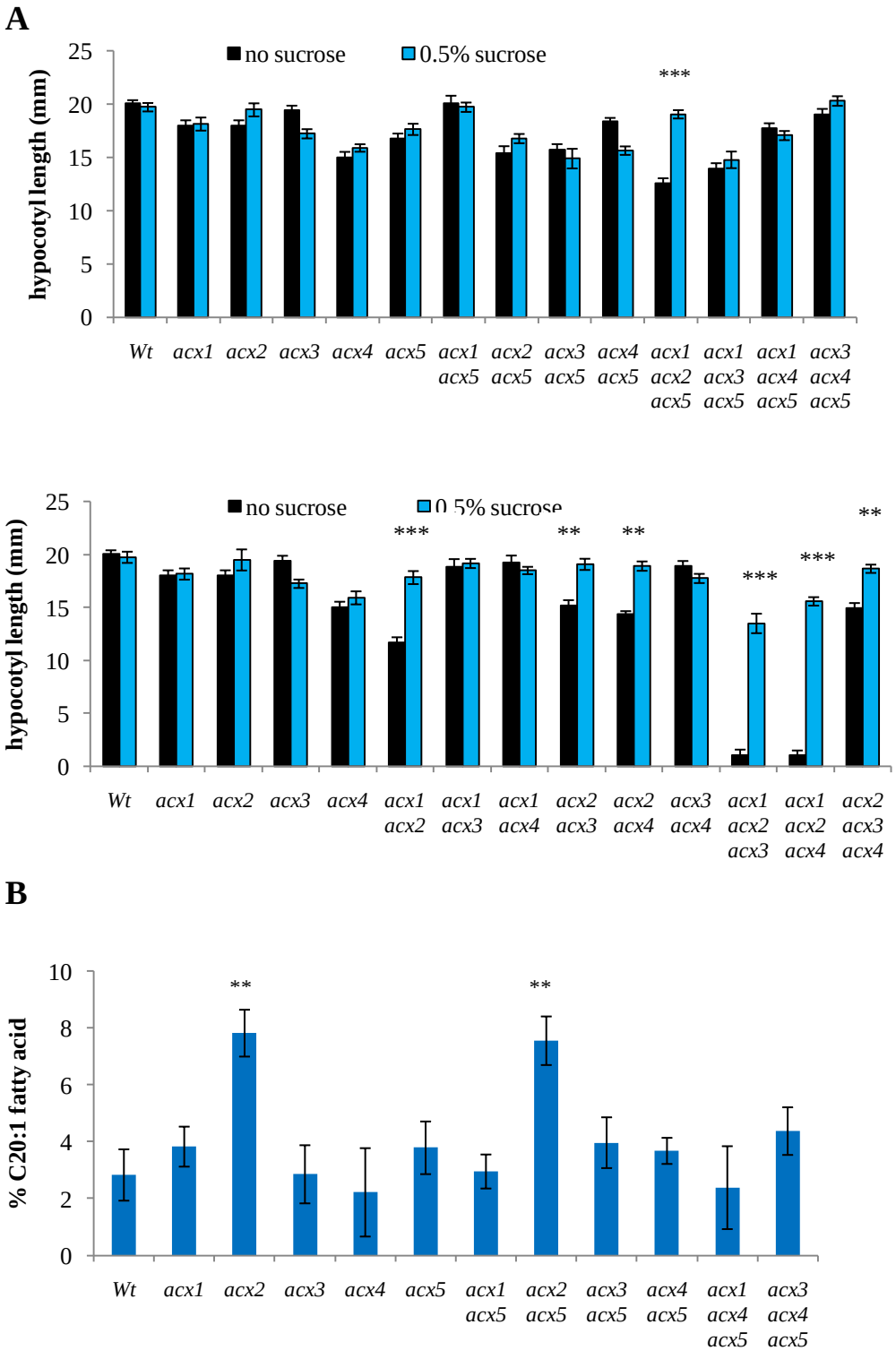
To investigate whether fatty acid β-oxidation is compromised in *acx1acx2acx3*, *acx1acx2acx4*, *acx1acx2acx5*, *acx1acx3acx5*, *acx1acx4acx5*, *acx2acx3acx4*, *acx2acx3acx5* and *acx3acx4acx5* I grew wild-type, single, double, and triple mutant seeds in the dark in the presence or absence of sucrose. *acx1acx5*, *acx3acx5*, *acx4acx5*, *acx1acx3acx5*, *acx1acx4acx5*, *acx2acx3acx4* and *acx3acx4acx5* showed normal development on medium lacking sucrose (Fig. 4-2A). *acx2acx3*, *acx2acx4*, *acx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* triple mutants displayed decreased hypocotyl elongation on medium without sucrose and this defect was more severe in *acx1acx2acx3* and *acx1acx2acx4* (Fig 4-2A). The hypocotyl elongation defects observed in *acx2acx3*, *acx2acx4*, *acx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* suggests that these mutants are compromised in fatty acid β-oxidation and this defect is more severe in *acx1acx2acx3* and *acx1acx2acx4*. This data indicate that ACX1, ACX2, ACX3 and ACX4 are important for long-chain fatty acid β-oxidation during early seedling development.

GC analysis was used to directly quantify the fatty acid β-oxidation defect of seed-storage fatty acids in these triple mutants. The *acx* single and double mutants were also used in the GC analysis for comparison. Eicosenoic acid (C20:1) levels were measured in wild-type and mutant lines at 6 days after germination. The results showed that wild-type seedlings had 2.8% C20:1 (vs. total fatty acids) remaining at 6 days compared to 7.8%, 16.3%, 11.1%, 10.2%, 7.6%, 20.2%, 19.9%, and 12% in *acx2*, *acx1acx2*, *acx2acx3*, *acx2acx4*, *acx2acx5*, *acx1acx2acx3*, *acx1acx2acx4* and *acx2acx3acx4*

respectively (Fig. 4-2B). Although *acx2* and *acx2acx5* showed no hypocotyl elongation defects on media lacking sucrose (Fig. 4-2A), these mutants retained 5% and 4.8% more eicosenoic acid compared to Wt (Fig. 4-2B). The defects were similar in *acx2* and *acx2acx5*, indicating that only the *acx2* mutant is contributing to the fatty acid β-oxidation defects in the *acx2acx5* double mutant. In the *acx2acx3* and *acx2acx4* double mutants, *acx3* and *acx4* are contributing to the fatty acid β-oxidation defects, although the respective single mutants can β-oxidize fatty acids similar to Wt.

To determine whether the *acx* triple mutants were defective in germination, 100 homozygous seeds mutants and Wt seeds were sterilized and plated on media supplemented with 0.5% sucrose. The results indicated that *acx1acx2*, *acx1acx2acx3* and *acx1acx2acx4* displayed delayed germination rate compared to Wt (Fig 4-2C). This defect was more severe in *acx1acx2acx3* and *acx1acx2acx4* triple mutants than Wt. By day 5, the percent germination was 33% and 50% respectively (Fig. 4-2C). Stratification is sometimes used to break seed dormancy and stimulate seeds to germinate. Therefore, to determine whether this germination defect could be rescued by seed stratification prior to analyzing germination, seeds were sterilized and kept at 4^{o}C in water for 3 days. After 3 days at 4^{o}C, seeds were plated on media supplemented with 0.5% sucrose and germination was recorded. The results showed that germination rate was still delayed but there was an increase in the number of seeds that germinated by day 5 (Fig. 4-2C). By day 5, 61% and 77% of *acx1acx2acx3* and *acx1acx2acx4* seed germinated as compared to non-stratified seeds (Fig. 4-2C). This data suggests that *acx1acx2acx3* and *acx1acx2acx4* fatty acid β-oxidation pathway is severely compromised so these mutants did not have the

necessary energy to emerge from the seed coat, but this defect was partially rescued by stratification.



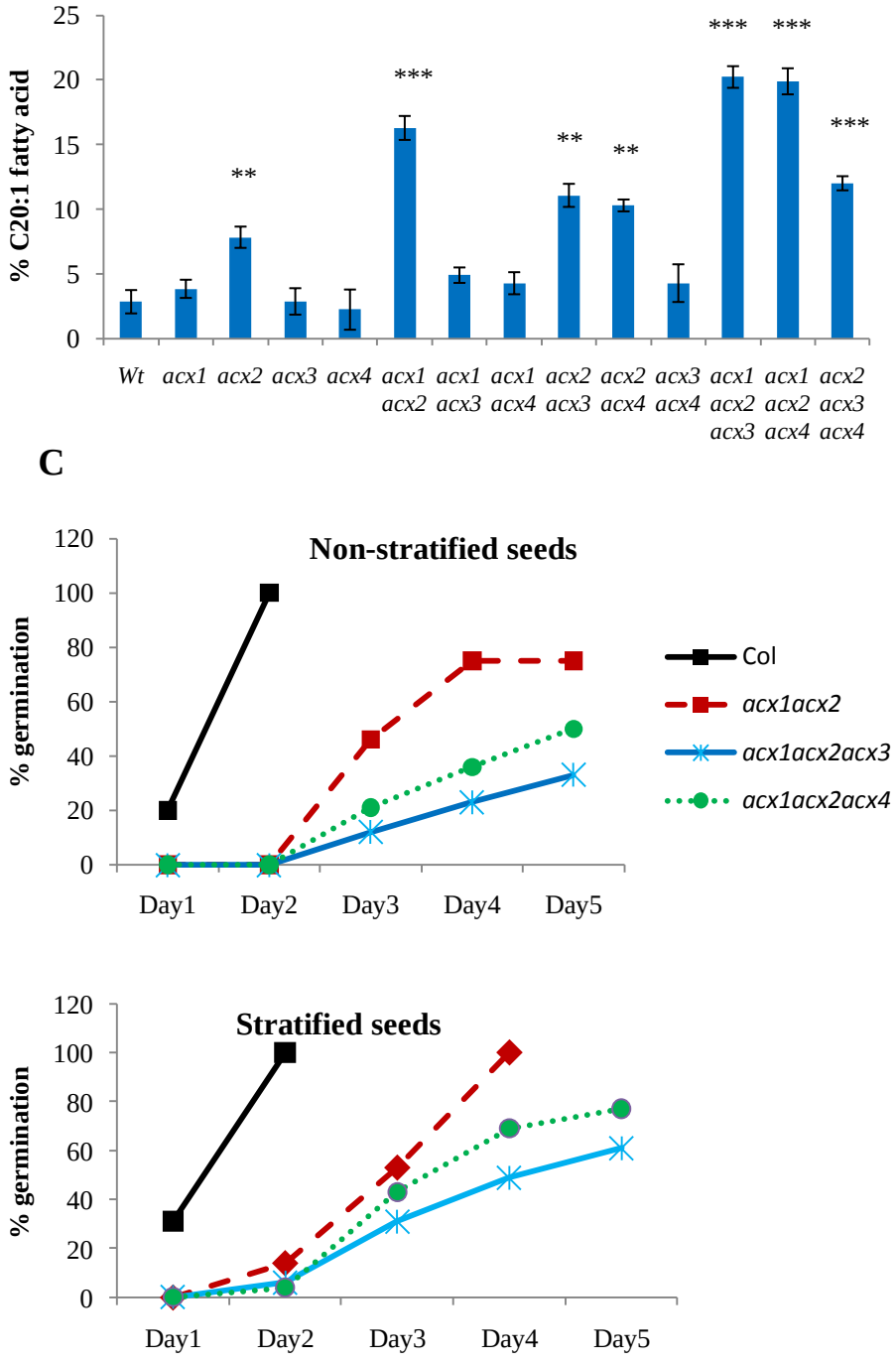


Fig. 4-2. Fatty acid metabolism in triple mutants.
(A) Hypocotyl length of wild-type Col-0 (Wt) and *acx* mutant seedlings grown 24 h in light and 6d in dark on medium with or without 0.5% sucrose. Error bars are the standard error of the means (n ≥ 12).(B) Percent of C20:1 fatty acids (vs. total fatty acids) in 6 d old light-grown seedlings. Error bars represent the standard error of the means of three biological replicates. (C) Germination rate of 100 seeds that were not stratified or stratified. Values of statistical significance relative to wild-type controls are indicated: ** $P < 0.01$, *** $P < 0.001$.

4.3. ACX activity in *acx* triple mutants

To further investigate the acyl-CoA defects in double and triple mutants, ACX activity was examined in these mutant extracts with various chain length fatty acyl-CoAs. The other triple mutants were not tested because seeds were not available. Acyl-CoA oxidase activity was not detected in *acx1acx2*, *acx1acx5, acx1acx2acx3, acx1acx2acx4* and *acx1acx4acx5* extracts on C18:1 acyl-CoA (Fig. 4-3A). *acx2acx3, acx2acx4, acx2acx3acx4* and *acx3acx4acx5* extract showed similar acyl-CoA activity as Wild-type on C18:1 acyl-CoAs (Fig. 4-3A). However, *acx2acx5, acx3acx5* and *acx4acx5* showed minor acyl-CoA oxidase defects compared to Wt (Fig. 4-3A). This result further confirmed that ACX1 is the sole acyl-CoA oxidase responsible for the β-oxidation of C18:1 but ACX5 may play a minor role.

Acyl-CoA oxidase activity was not detected in *acx1acx2acx3* extracts on C12:0 acyl-CoA (Fig 4-3B). However, detectable but significantly reduced acyl-CoA activity was observed in *acx2acx3*, *acx3acx5* and *acx3acx4acx5* extracts on this substrate (Fig. 4-3B). However, *acx3acx4* showed residual acyl-CoA activity on C12:0 acyl-CoA (Fig. 4-3B). Minor inhibition of acyl-CoA activity was detected in *acx1acx2*, *acx1acx5*, *acx1acx2acx4* and *acx1acx4acx5* (Fig. 4-3B). These confirmed that ACX1 and ACX3 have overlapping substrate specificity. ACX3 is most active on C12:0, but ACX1 can partially compensate for loss of ACX3.

Significantly reduced acyl-CoA activity was observed in *acx2acx4, acx4acx5, acx1acx2acx4, acx1acx4acx5, acx2acx3acx4* and *acx3acx4acx5* (Fig. 4-3C). *acx1acx2,*

acx2acx3 and *acx1acx2acx3* showed similar activity to Wt on C6:0 acyl-CoA (Fig. 4-3C). *acx1acx5*, *acx2acx5* and *acx3acx5* showed a minor reduction in acyl-CoA activity on C6:0 acyl-CoA (Fig. 4-3C). Interestingly, this observation suggests that perhaps ACX5 can partially metabolize short chain fatty acids. This observation is unexpected because we assume that ACX5 may act like ACX1 given their high degree of similarity. Taken together, this data indicate that ACX4 is the major acyl-CoA oxidase involved in the metabolism of short chain fatty acid substrates.

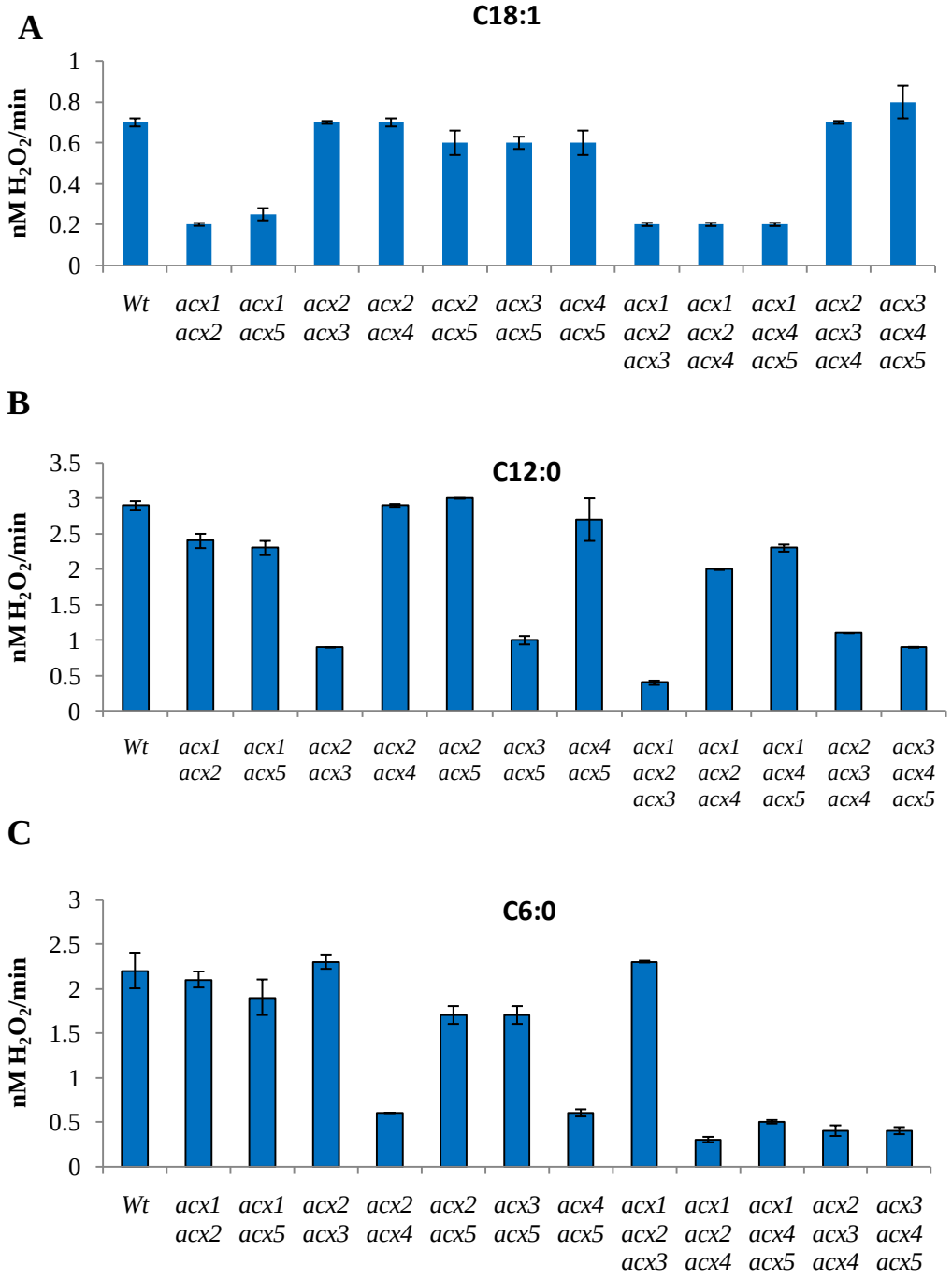


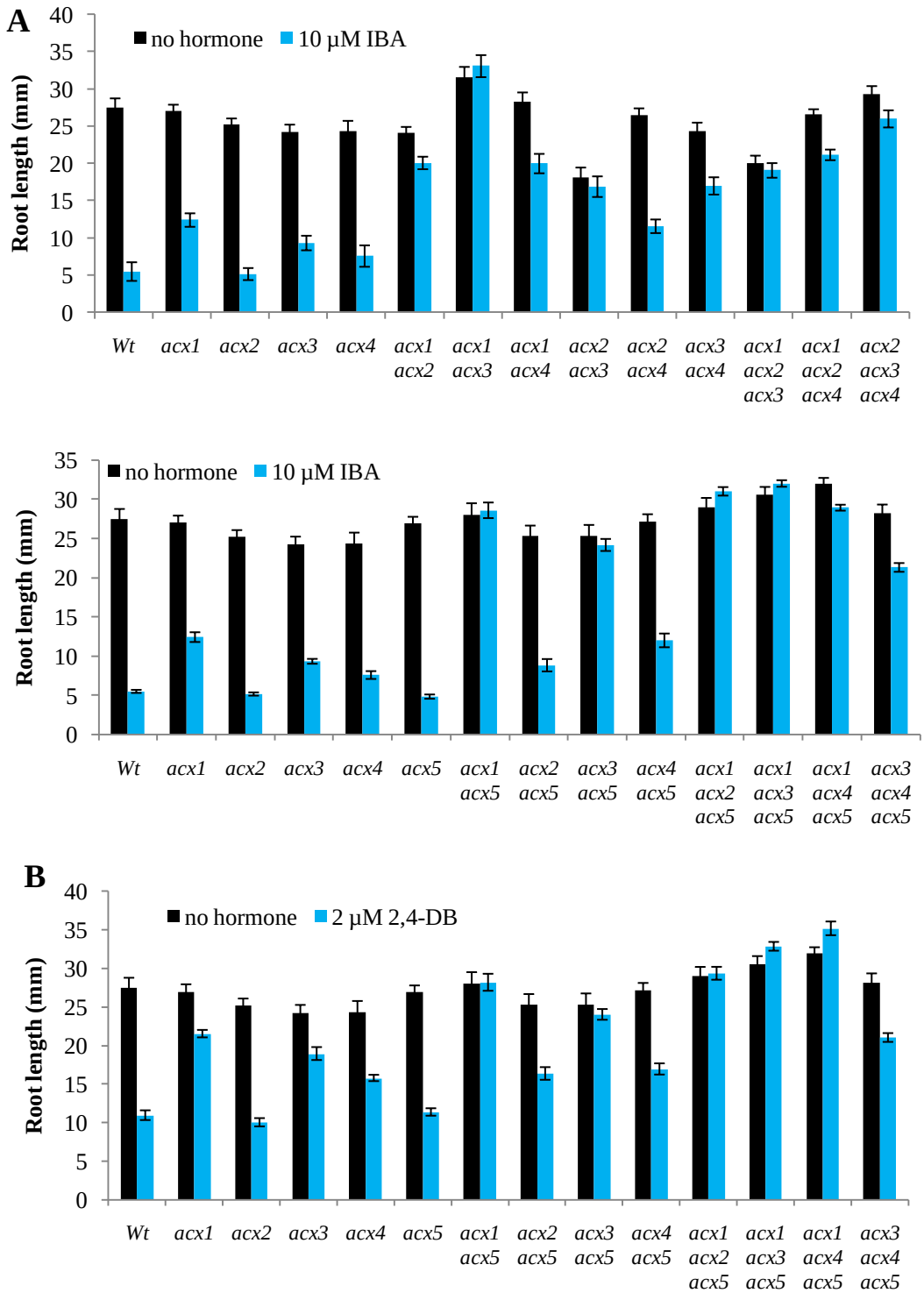
Figure 4-3. Acyl-CoA oxidase activity of *acx* mutants with fatty acid substrates
Extracts from 3 d old light grown Col-0, *acx1acx2*, *acx1acx5*, *acx2acx3*, *acx2acx4*, *acx2acx5*, *acx3acx5*, *acx4acx5*, *acx1acx2acx3*, *acx1acx2acx4*, *acx1acx4acx5*, *acx2acx3acx4* and *acx3acx4acx5* seedlings were tested for acyl-CoA oxidase activity with the following fatty acid substrates (A) oleoyl-CoA (C18:1), (B) lauroyl-CoA (C12:0), and (C) n-hexanoyl-CoA (C6:0). Data are shown in nM H_2O_2 produced per minute. Error bars = standard deviations of rates of three independent experiments.

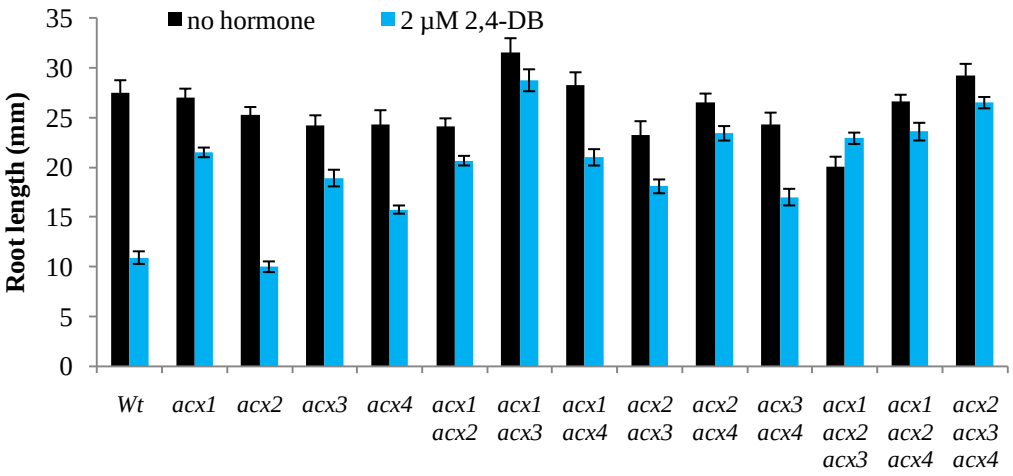
4.4. Responses to auxin and propionate

To determine whether the double and triple mutants are defective in IBA to IAA conversion, wild type and *acx* mutants were assayed on inhibitory concentrations of IBA or the IBA analog 2,4-DB. All of the single mutant and higher-order mutants displayed decreased sensitivity to the inhibitory effects of IBA and 2,4-DB on root elongation compared to wild type (Fig. 4-4A, B). The *acx1acx2, acx1acx3, acx1acx5, acx2acx3, acx3acx5, acx1acx2acx3, acx1acx2acx4, acx1acx2acx5, acx1acx3acx5, acx1acx4acx5* and *acx2acx3acx4* seedlings were more IBA and 2,4-DB resistant than the other mutant combinations (Fig. 4-4A, B). To determine whether this response was specific to IBA and IBA analogs these mutants were assayed on IAA. The result showed that all the mutants were sensitive to IAA. These results suggest that the *acx* mutants are compromised specifically in IBA (and 2,4-DB) β-oxidation and not IAA transport or signaling. Taken together the data indicate that the ACXs play a role in IBA β-oxidation.

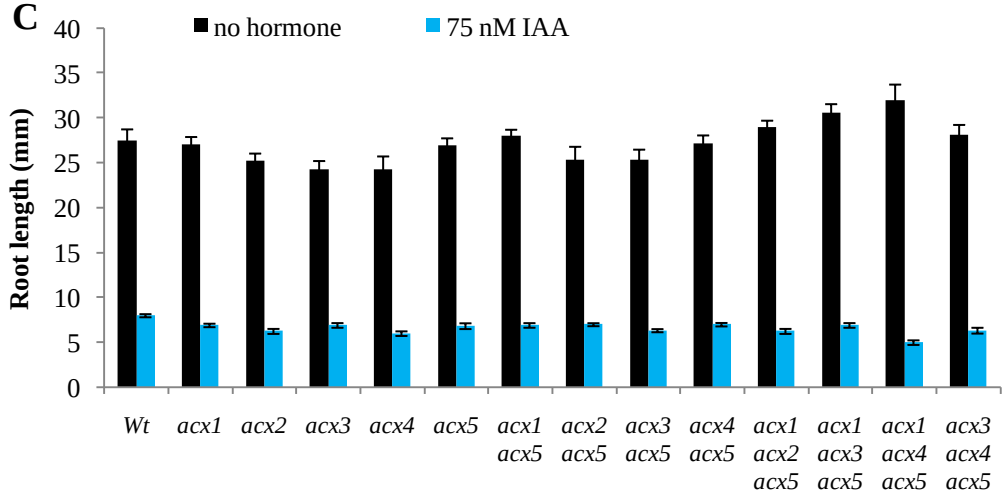
To determine whether the combined loss of *ACX1, ACX2, ACX3, ACX4* and *ACX5* affects propionate processing, Col-0, *chy1-3*, and *acx* mutant seeds were assayed on exogenous propionate. The *chy1* mutant showed strong inhibition (Fig. 4-4C). Root elongation of *acx* mutants all showed a similar degree of weak sensitivity to propionate, except for *acx4* and mutant combinations containing *acx4* (Fig. 4-4C). These mutants were more sensitive to propionate than Wt (Fig. 4-4C). *acx2* and *acx5* showed similar root elongation to Wt on propionate and mutant combinations containing these two mutants did not show enhanced sensitivity on this media (Fig. 4-4C). These results suggest that

ACX1, ACX3, ACX4 but not ACX2 or ACX5 may play partial or overlapping roles in propionate metabolism.




no hormone
2 µM 2,4-DB
Root length (mm)
35
30
25
20
15
10
5
0
Wt
acx1
acx2
acx3
acx4
acx1 acx2
acx1 acx3
acx1 acx4
acx2 acx3
acx2 acx4
acx3 acx4
acx1 acx2 acx3
acx1 acx2 acx4
acx2 acx3 acx4

C


no hormone
75 nM IAA
Root length (mm)
40
35
30
25
20
15
10
5
0
Wt
acx1
acx2
acx3
acx4
acx5
acx1 acx5
acx2 acx5
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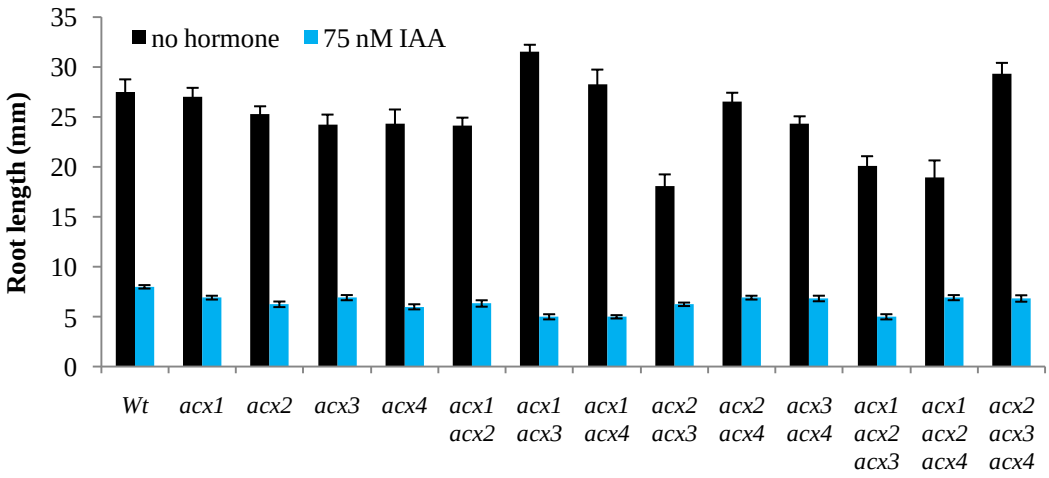
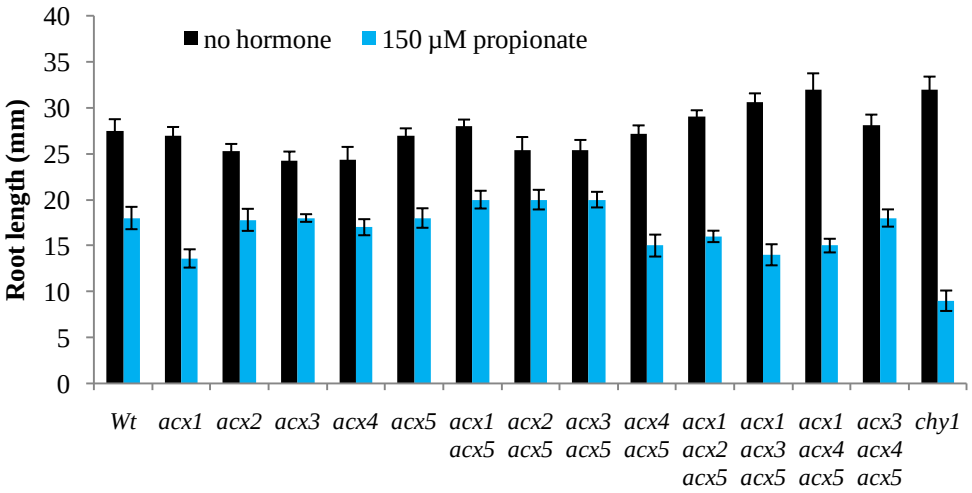
acx3 acx4 acx5


no hormone
75 nM IAA
Root length (mm)
35
30
25
20
15
10
5
0
Wt
acx1
acx2
acx3
acx4
acx1 acx2
acx1 acx3
acx1 acx4
acx2 acx3
acx2 acx4
acx3 acx4
acx1 acx2 acx3
acx1 acx2 acx4
acx2 acx3 acx4

D



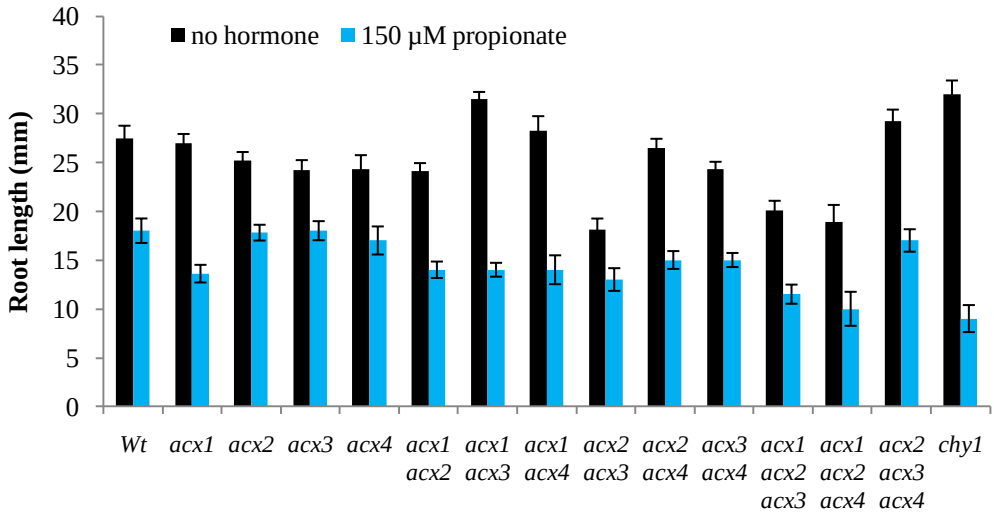


Fig. 4-4. *acx* mutants responses auxins and propionate.
Root lengths of 8 d old Col-0 (Wt) and mutant seedlings grown under continuous white light on 0.5% sucrose medium with (A) 10 µM IBA, (B) 2 µM 2,4-DB, (C) 75 nM IAA and (D)150 µM propionate. *chy1-3* was used as a control in the propionate experiments to show a sensitive phenotype (Lucas et al., 2007). Error bars represent the standard error of the means ($n \geq 12$). Values of statistical significance relative to wild-type controls are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.5. Adult phenotype

Observation of *acx1acx2acx3*, *acx1acx2acx4*, *acx1acx2acx5* and *acx1acx3acx5* adult plants showed that these plants had a reproduction phenotype. *acx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* produced significantly fewer seeds than Wt. The *acx1acx2acx5* plants had a more dramatic adult phenotype. The adult plants were smaller and the number of seeds produced by this mutant was significantly reduced compared to *acx1acx2acx3* and *acx1acx2acx4*. Measurements of siliques and weights of the seeds showed that mutant siliques were smaller than Wt and seeds weight less than Wt (Fig. 4-5A, B). This suggests that *acx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* are defective in reproduction. Siliques of *acx1acx3acx5* plants only elongate and produce seeds when plants were dipped in JA solution. The observation that *acx1acx3acx5* plants only produced seeds when dipped in JA suggests that these plants were defective in the JA biosynthesis pathway. *acx1acx2acx5* plants produced slightly more seeds when dipped in JA than plants not dipped. However, there was no difference in the amount of seeds recovered when *acx1acx2acx3* and *acx1acx2acx4* were dipped in JA as compared to plants that were not dipped. These observations suggest that the reproductive defect in *acx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* is not due only to a defect in the JA pathway.

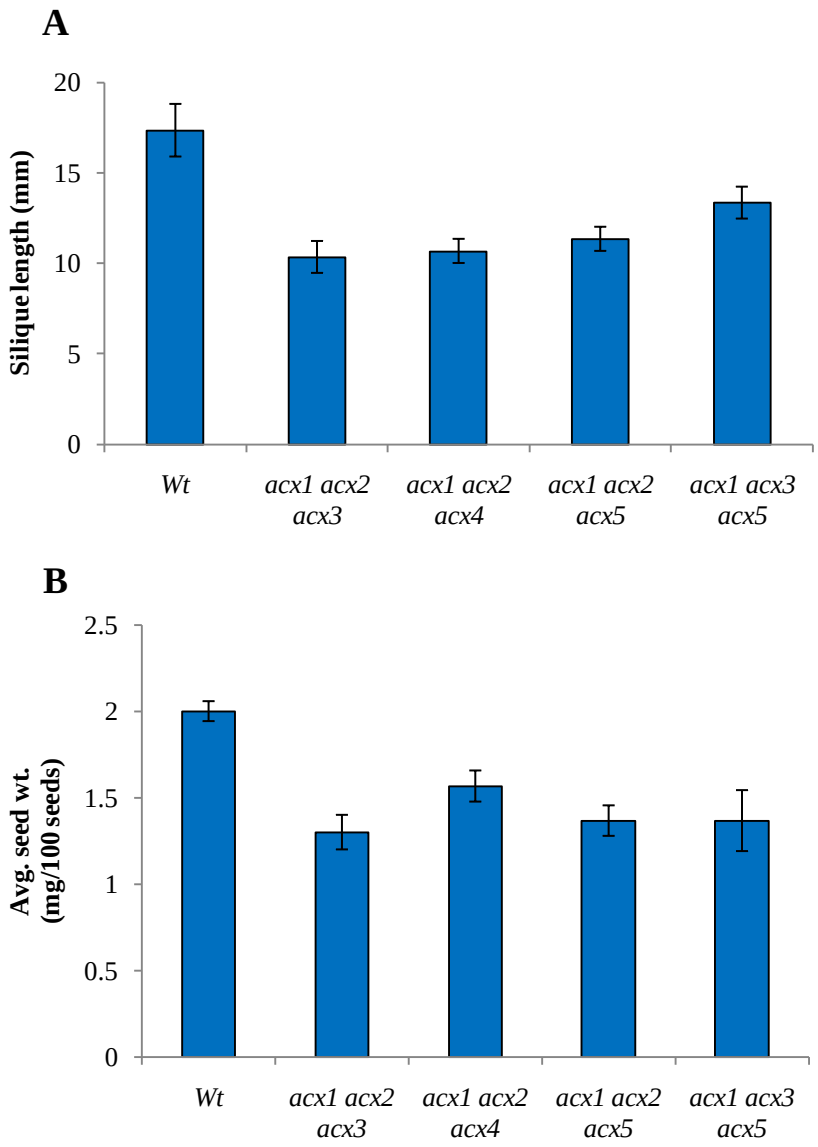


Figure 4-5. *acx* fertility defects.

(A) Length of 5th silique on the main stem. Error bars represent the standard error of the means ($n \geq 8$).

(B) Average seed weight in mg per 100 seeds. Error bars represent the standard error of the means ($n \geq 4$).

4.6. Discussion

Arabidopsis has six acyl-CoA oxidase enzymes, five of which appear to play roles in β-oxidation pathways. *ACX1-ACX4* all are expressed throughout many stages of plant growth and development with somewhat unique, but largely overlapping, expression patterns. The results of many previous studies indicate that ACX2 prefers very-long-chain substrates (maximum activity on C18), ACX1 works on long-chain substrates (C14), ACX3 catalyzes medium-chain substrates (C12), and ACX4 acts on short-chain substrates (C6). However, the enzymes show substantial overlap in their substrate ranges, particularly ACX1 (C8-C20) and ACX3 (C8-C14). Although each enzyme shows activity on specific substrates, their activities in enzyme assays are not necessarily comparable. For instance, *in vitro* assays done in parallel showed ACX1 and ACX3 catabolized overlapping substrates, but the specific activity of ACX3 was much higher than ACX1 (Froman et al., 2000).

Combining the expression information and *in vitro* assays provides a glimpse into the activity of these enzymes, but to ascertain the role of each protein in a plant, we must consider the questions of functional redundancy and cross-regulation of gene expression. Based on these complications, it is important to study these proteins in plants to determine the specific role that each enzyme plays during plant growth and development.

Previous studies revealed that mutants with disruptions in individual *ACX* genes (*acx1-acx5*) showed few phenotypes. Each *acx* mutant in the Col-0 background had wild-type growth in the absence of sucrose, indicating that normal fatty acid metabolism levels

were maintained (Adham et al., 2005). *acx4* single mutants had an altered development in the absence of sucrose in the light, but had normal responses in the dark (Adham et al., 2005), indicating that the mutant may have a specific defect under certain environmental conditions. Biochemical studies revealed that loss of ACX3 and ACX4 resulted in dramatic, but incomplete reduction of acyl-CoA oxidase activity on C6 and C12 fatty acid substrates, respectively (Adham et al., 2005). These results suggest that other ACX enzymes can partially compensate for loss of ACX3 and ACX4. However, loss of ACX1 resulted in complete reduction of acyl-CoA oxidase activity on the C18:1 fatty acid substrate (Adham et al., 2005), indicating that the other ACX enzymes are not as able to compensate for loss of ACX1.

In this chapter, I used additional *acx* triple mutants to define the roles of *ACX2* and *ACX5* in peroxisomal β-oxidation pathways. I isolated and characterized *acx1acx2acx3*, *acx1acx2acx4*, *acx1acx2acx5*, *acx1acx3acx5*, *acx1acx4acx5*, *acx2acx3acx4*, *acx2acx3acx5*,*acx2acx4acx5* and *acx3acx4acx5* triple mutants in Arabidopsis Col-0 accession. An *acx1acx2* double mutant showed significant defects in fatty acid β-oxidation in the light and the dark (Adham et al., 2005; Pinfield-Wells et al., 2005), revealing a strong role for ACX1 and ACX2 in seed-storage metabolism that is only partially compensated by ACX3. I showed here that this growth defect reflects reduced degradation of the seed-storage fatty acid C20:1 (Fig. 4-2B). This phenotype is consistent with the characterized activity of ACX1 and ACX2 in oxidizing long-chain substrates *in vitro*. I found that although the *acx2* single mutant did not show a defect in hypocotyl elongation on media lacking sucrose, this mutant retained more C20:1 fatty acid at day 6

compared to wild-type (Fig. 4-2A, B). The *acx2acx3*, *acx2acx4* and *acx2acx5* double mutants retained C20:1 fatty acid similar to the *acx2* single mutant (Fig. 4-2B). This showed that ACX2 is making a significant contribution to fatty acid β-oxidation in Arabidopsis Col-0 accession. *acx1acx2acx3* and *acx1acx2acx4* were defective in fatty acid β-oxidation, as shown by hypocotyl elongation, GC analysis and germination rate (Fig. 4-2). This further showed the importance of ACX2 in fatty acid β-oxidation, as *acx1acx3* and *acx1acx4* double mutants β-oxidized fatty acids similar to wild-type (Fig. 4-2A, B). *acx2* is the only single mutant that retained seed storage fatty acids. Thus, in mutant combinations with *acx1*, *acx3*, *acx4* and *acx5*, ACX2 may be compensating for the loss of these genes.

The *acx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* triple mutant plants were smaller than wild-type. They all make smaller siliques, fewer and smaller seeds than wild-type (Fig. 4-5). However, the number of seeds produced by *acx1acx2acx5* was significantly less than *acx1acx2acx3* and *acx1acx2acx4*. When a*cx1acx2acx5* plants were treated with jasmonic acid they did not produce more seeds than non-treated plants. This indicated that the reduced fertility in *acx1acx2acx5* is due to a fatty acid defect and not a JA biosynthesis defect. The reduced fertility in this mutant may be due to accumulation of toxic intermediates that inhibit fertility, since the *acx1acx2acx5* seedlings showed enhanced sensitivity to propionate (Fig. 4-4 D). ACX2 is also contributing to IBA β-oxidation. The *acx2* single mutant was sensitive to IBA and 2,4-DB, but *acx1acx2*, *acxacx1acx2acx3*, *acx1acx2acx4* and *acx1acx2acx5* showed enhanced IBA and 2,4-DB

insensitivity compared to their respective single and double mutant parents (Fig. 4-4A, B). This showed a role for ACX2 in IBA β-oxidation.

In this study, I show that *acx1acx5*, *acx2acx5* and *acx3acx5* had reduced acyl-CoA oxidase activity on C6:0 fatty acid. This suggests a role for ACX5 in fatty acid β-oxidation (Fig. 4-3C). Further, *acx1acx5*, *acx2acx5*, *acx3acx5*, *acx4acx5*, *acx1acx2acx5*, *acx1acx3acx5*, *acx1acx4acx5* and *acx3acx4acx5* showed enhanced IBA and 2,4-DB insensitivity compared to their respective single and double mutant parents (Fig. 4-4A, B). ACX1 and ACX5 play a role in JA synthesis (Schilmiller et al., 2007). The *acx1acx5* double mutant showed reduced fertility but produced seeds without treatment with JA. However, the *acx1acx3acx5* triple mutant only produced seeds were the plants were treated with JA. This showed that ACX3 is contributing to JA synthesis. This observation will be confirmed by quantifying JA in *acx1acx3acx5* compared to *acx1acx5*. Taken together, these data show a role for ACX5 in other peroxisomal β-oxidation processes. Cloning of *ACX5* and *in vitro* protein assays with the ACX5 protein will elucidate the role of ACX5 in fatty acid β-oxidation. Thus, the triple mutants in this study showed a role for both ACX2 and ACX5 in fatty acid β-oxidation and other peroxisomal β-oxidation processes.

CHAPTER 5: *pex5* mutants that differentially disrupt PTS1 and PTS2 peroxisomal matrix protein import in Arabidopsis.

The PEX5 and PEX7 receptors are required for import of peroxisome matrix-bound proteins. Peroxisome matrix proteins contain one of two peroxisomal targeting signals (PTS1 or PTS2). The PEX5 receptor imports PTS1 proteins, while PEX7 imports PTS2 proteins. However, PEX7 binds to PEX5 to import PTS2 proteins. In this chapter, I used different *pex5* mutants to separate PTS1 and PTS2 protein import, to examine the role of these proteins in specific peroxisomal processes. This work was e-published in October.

5.1. PEX5 protein accumulation.

I began studying PEX5 by investigating how the protein accumulates in plants. Western blots using an antibody that recognizes the C-terminus of PEX5 (Zolman and Bartel, 2004) revealed that the protein accumulates at high levels in seedlings and at lower levels as plants mature (Fig. 5-1B). Because peroxisomal metabolism, and hence PEX5, is critical during times of early seedling development when fatty acids are being highly metabolized, I also checked accumulation of the protein under different developmental conditions. PEX5 is present at a higher level in the dark than in the light (Fig. 5-1C). In addition, I saw higher accumulation in the absence of sucrose than when sucrose was included in the media (Fig. 5-1C). These protein patterns are consistent with increasing β-oxidation during dark development when photosynthesis cannot occur and decreases when sucrose is present, indicating photosynthesis is occurring. These protein studies are consistent with publically available microarray data, which shows that *PEX5* gene

expression is highest in seeds but relatively stable throughout plant development (Fig. 5-1D).

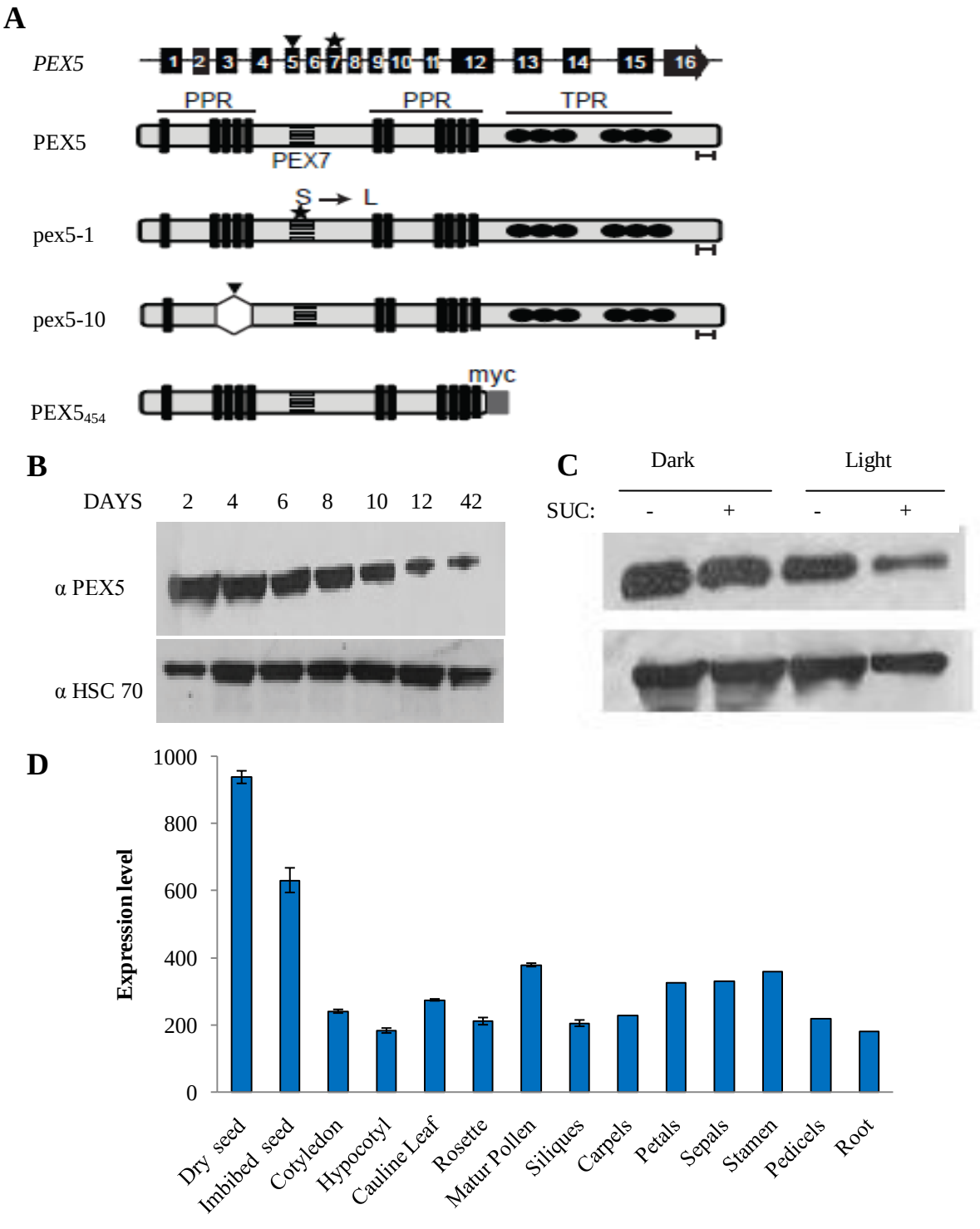


Figure 5-1. *PEX5* gene expression and protein accumulation.

(A) Cartoon representation of the *PEX5* gene and the wild-type PEX5 protein, the pex5 mutant proteins, and the $PEX5_{454}$ truncation. The *pex5-1* point mutation (Zolman et al., 2000) and *pex5-10* insertions are indicated by a star and a triangle, respectively, with the resulting protein alterations shown below. Horizontal lines indicate the PEX7 binding domain (PEX7), circles indicate the tetratricopeptide repeat (TPR) domains, and rectangles represent the pentapeptide repeat (PPR) domains. The goalpost under the protein indicates the recognition site of the PEX5 antibody (Zolman and Bartel, 2004).

(B) Total protein was purified from wild-type Col-0 seedlings and adult plants and analyzed by western blot for PEX5 accumulation.

(C) Total protein from 5 d old wild-type seedlings grown in the dark or light in the presence or absence of sucrose was analyzed by western blot for PEX5 accumulation. For both B and C, equal protein loading was examined by incubation with HSC70 antibody**.**

(D) Levels of *PEX5* mRNA expression in dry and imbibed seeds, cotyledons, hypocotyls, cauline leaves, rosette leaves, mature pollen, seeds/siliques, carpels, petals, and roots. The graph was constructed using Arabidopsis eFP Browser data from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007). Data was retrieved on March 6th, 2010.

5.2. *pex5-10* makes an altered *pex5* protein.

To study the role of PEX5 in plants and examine the effects of peroxisomal import during plant development, I wanted to examine a stronger mutant allele than the previously-described *pex5-1* point mutation (Zolman et al., 2000). The *pex5-10* mutant has a T-DNA insertion in exon 5 (Fig. 5-1A), which I confirmed by PCR amplification using genomic and T-DNA specific primers (Fig. 5-2A). Sequence analysis of the mutant line revealed that the site of the insertion was at position 1769 (with 1 being the A of the ATG initiator). Using Western blots with a PEX5 antibody, I detected no full-length protein in the mutant (Fig. 5-2B). However, I saw a lower molecular-weight product (Fig. 5-2B), which segregated in an F_1 backcross plant (Fig. 5-2C). The smaller protein product was typically present at a lower abundance, but consistently was detectable through several generations and with additional back-crosses to wild type (Fig. 5-2C). To determine the protein sequence, I isolated cDNA from the mutant line and did RT-PCR using *PEX5*-specific primers. I did not see a product using primers directly surrounding the insertion, consistent with our PCR data. However, I could see products amplified using either upstream or downstream primer sets (Fig. 5-2D). Although I could not amplify products from primers in the same exon as the insertion, I could amplify smaller products using spanning primers that flanked the insertion (Fig. 5-2D). I sequenced the spanning product and found a complete deletion of exon 5 along the normal exon/intron junctions in the mutant cDNA, with the insertion removed. This exclusion results in production of a mutant protein lacking four of the pentapeptide repeats (Fig. 5-1A). The abnormal protein accumulated in both leaves and roots of adult plants at similar levels, indicating this alteration was not tissue specific (Fig. 5-2B).

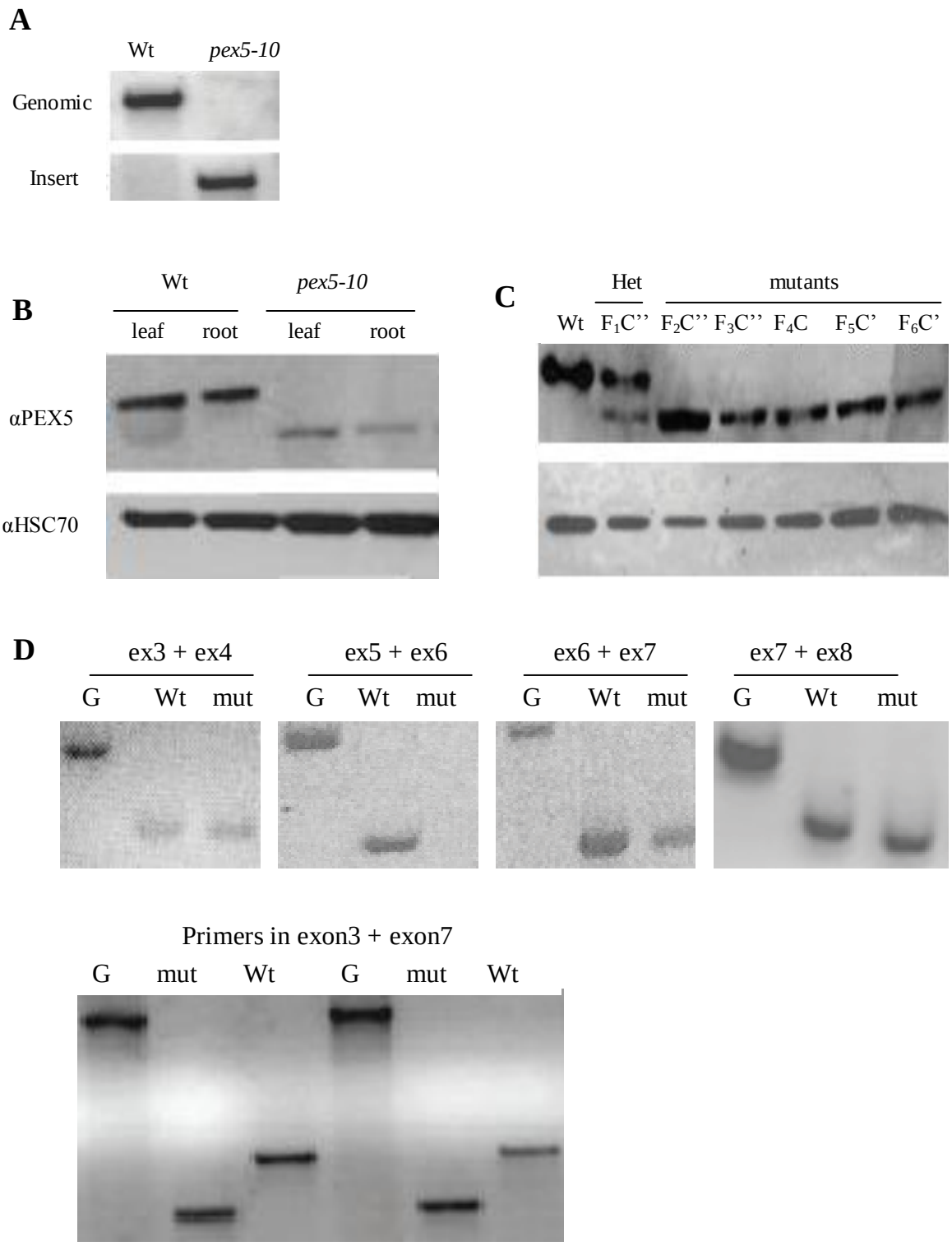

A
Wt
pex5-10
Genomic
Insert
B
Wt
pex5-10
leaf
root
leaf
root
αPEX5
αHSC70
C
Het
mutants
Wt
F1C''
F2C''
F3C''
F4C
F5C'
F6C'
D
ex3 + ex4
ex5 + ex6
ex6 + ex7
ex7 + ex8
G
Wt
mut
Primers in exon3 + exon7
G
mut
Wt
G
mut
Wt

Figure 5-2. T-DNA insertion effects in the *pex5-10* mutant.

(A) DNA was extracted from wild-type and *pex5-10* plants and PCR-amplified with *PEX5* gene-specific (genomic) and T-DNA (insertion) primers (Supplemental Table 1).

(B) Total protein extracted from leaves and roots of 12 d old wild-type and *pex5-10* seedlings was analyzed by western blot with a PEX5 antibody generated against the C-terminus of the PEX5 protein.

(C) Total protein from 3 week old wild-type and *pex5-10* plants was analyzed by western blot for PEX5 accumulation. Seedlings were taken from the heterozygous progeny of a *pex5-10* backcross to Col-0 (F_1c'') and *pex5-10* homozygous mutant plants over four generations (F_2 through F_6) and three backcrosses (F_4c for the first backcross; F_5c', F_6c' for the second backcross; F_2c'', F_3c'' for the third backcross).

For all blots, protein loading was determined by incubation with HSC70 antibody.

(D) cDNA was synthesized from RNA isolated from 3 wk old wild-type (Wt) and *pex5-10* (mut) plants. To examine the effects of the T-DNA insertion in exon 5, cDNAs from wild-type and *pex-10* plants were amplified with *PEX5* primers that bind upstream (exons 3+4), directly surrounding (exons 5+6), or downstream (exons 6+7) and (exons7 + 8) of the insert. The spanning primers are in exons 3 + 7. Genomic DNA (G) from wild-type plants was amplified with all primer sets to show purity of cDNA synthesis. Primers are listed in Table 2-2.

5.3. *pex5-10* import defects and a PTS1 specific mutant.

One rationale for studying *pex5* mutants is to determine the effects of losing PTS1 import into peroxisomes. However, the *pex5-1* mutant has a PTS2 specific defect (Woodward and Bartel, 2005). In contrast, *pex5-10* is defective in both PTS1 and PTS2 peroxisomal import; the mutant cannot efficiently import thiolase, a PTS2 protein (Zolman et al., 2005; Lingard and Bartel, 2009; Ramón and Bartel, 2010), catalase (Lingard and Bartel, 2009; Lingard et al., 2009), isocitrate lyase (Lingard et al., 2009), malate synthase (Lingard and Bartel, 2009), and peroxisomal malate dehydrogenase (Lingard and Bartel, 2009; Ramón and Bartel, 2010). In addition, the reporter constructs GFP-SKL (Lee et al., 2006), and PTS2-GFP (Lee et al., 2006; Lingard and Bartel, 2009) show cytosolic mislocalization. PEX5 RNAi lines have similar disruptions in both PTS1 and PTS2 import (Hayashi et al., 2005).

To characterize the effects of *pex5-1* and *pex5-10* import defects on peroxisomal function, I measured the activity of two acyl-CoA oxidase (ACX) enzymes, ACX3 and ACX4. These enzymes act in the first step of peroxisomal fatty acid β-oxidation (Graham, 2008). ACX3 has a PTS2 signal, while ACX4 has a PTS1 signal. *In vitro* and *in vivo* activity assays indicate that ACX3 primarily β-oxidizes medium-chain fatty acids, such as C12:0 (Eastmond et al., 2000; Froman et al., 2000; Adham et al., 2005) and ACX4 β-oxidizes short-chain substrates of C4:0 – C6:0 fatty acids (Hayashi et al., 1999; Rylott et al., 2003; Adham et al., 2005). Extracts prepared from *pex5-1* had similar activity on C6:0 substrates as wild type, but *pex5-10* showed significantly reduced activity on this substrate (Fig. 5-3A). This data confirms that PTS1 protein activity is not

disrupted in the *pex5-1* mutant but is severely compromised in the *pex5-10* mutant. The *pex5-1* and *pex5-10* mutants were both impaired in β-oxidation of C12:0 fatty acid substrates, confirming that these mutants have disruptions in PTS2 import (Fig. 5-3A). To determine the defects in *pex5-1* and *pex5-10* in a more direct manner I visualized homozygous mutants and Wt plants transformed with GFP, GFP PTS1 and GFP PTS2 constructs with a confocal microscope. These constructs contain GFP tagged with PTS1 or PTS2 signals; if these proteins are imported optimally then the GFP signal will be seen in a punctate pattern. The results showed *pex5-1GFP PTS1* had similar PTS1 protein import as Wt, but PTS2 protein import was reduced when *pex5-1GFP PTS2* was observed (Fig. 5-3B). Import of both *GFP PTS1* and *GFP PTS2* was reduced in *pex5-10* mutants (Fig. 5-3B).

To investigate the role of PEX5 in more detail, I attempted to artificially create a plant line with a PTS1 specific import defect. I introduced six constructs that expressed varying regions of the PEX5 protein into wild-type plants. However, five of the mutant proteins did not accumulate in wild-type plants selected to contain the transgene. The $PEX5_{454}$ construct encodes the first 454 amino acids of the protein, including the PPR repeats important for peroxisomal membrane protein binding and the PEX7 interaction domain; the construct does not have any of the TPR domains known to bind the PTS1 signal of peroxisome-bound proteins (Fig. 5-1A). Because the PEX5 antibody recognizes the C-terminus of the full-length protein, I added the myc epitope tag to the C-terminus of $PEX5_{454}$. I transformed the $PEX5_{454}$ construct into wild-type Arabidopsis and analyzed transformants for mutant protein accumulation (Fig. 5-3C). Col-0 ($PEX5_{454}$) plants were

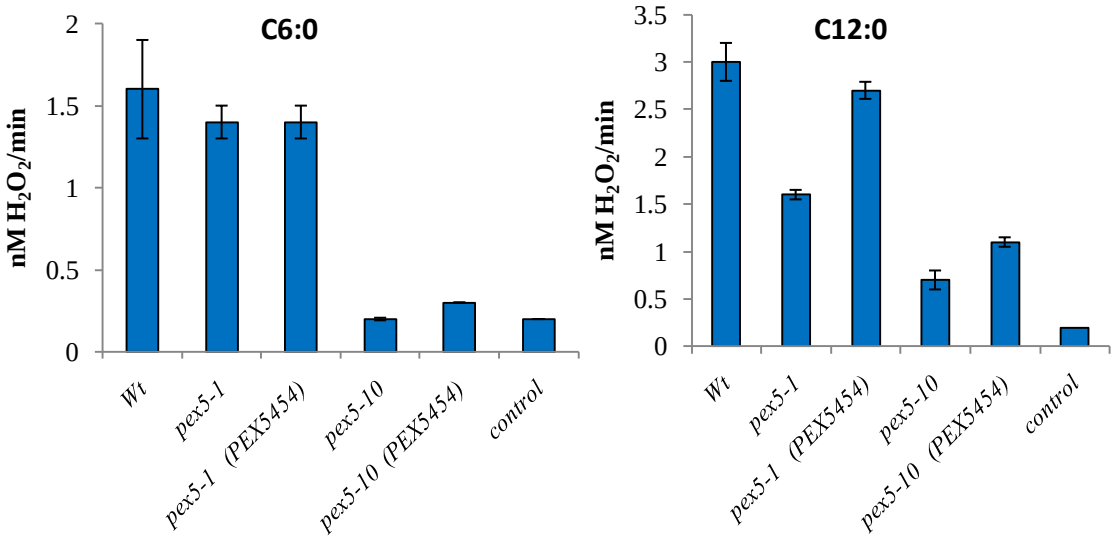
then crossed to *pex5-1* and *pex5-10* mutants. In part, this strategy was utilized because of the low germination of *pex5-10* seeds, especially on herbicide. I selected mutant lines containing the transgene by PCR, then confirmed the accumulation of the $PEX5_{454}$ protein by western blot (Fig. 5-3C). Because I saw some variability in *pex5-10* (*$PEX5_{454}$*) line responses, four independent lines (designated p109, p111, p122, p126) are shown for most assays to represent the range of responses.

The hypothesis was that $PEX5_{454}$ would restore PTS2 import in the *pex5* mutants, given that the $PEX5_{454}$ protein retains the regions necessary for PEX7 binding and peroxisomal membrane/import. To determine the effects of the transgene on PTS2 import, I examined processing of the PTS2 protein 3-ketoacyl-CoA thiolase, an enzyme that acts in the final step of peroxisomal fatty acid β-oxidation (Hayashi et al., 1998). PTS2 proteins are synthesized in the cytosol, and the 26-amino acid N-terminal signal sequence is proteolytically cleaved following import into the peroxisomal matrix, resulting in a smaller mature protein (Hijikata et al., 1987; Schuhmann, 2008). Analysis of thiolase processing from the larger cytosolic form to the smaller mature form by Western blot can therefore reveal the degree of peroxisomal import. Both *pex5-1* and *pex5-10* showed defects in thiolase processing, with *pex5-10* being more severe (Fig. 5-3D). However, inclusion of the *$PEX5_{454}$* transgene in *pex5-1* mutants led to a complete rescue of the defect in thiolase processing. The *pex5-10 ($PEX5_{454}$)* lines were all still defective to varying degrees in thiolase processing, but showed improvement compared to the parent phenotype (Fig. 5-3D). These results suggest that the $PEX5_{454}$ protein can improve PTS2

import in both mutant plants. *pex5-10* complementation with a full-length *PEX5* cDNA fully rescued the mutant defects (Fig. 5-3D).

To examine the effects of this rescue on peroxisomal function, I again tested ACX activity. The *pex5-1* mutant was defective in C12:0 metabolism, but addition of $PEX5_{454}$ rescued this activity to wild-type levels (Fig. 5-3A). The *pex5-10* mutant was severely impaired in C12:0 fatty acid oxidation, which was partially rescued by the $PEX5_{454}$ protein (Fig. 5-3A), similar to the partial increase in thiolase processing (Fig. 5-3D). This data suggests that the *pex5* PTS2 import defects are reduced by inclusion of the $PEX5_{454}$ protein. The activity assays showed similar C6:0 fatty acid activities in *pex5-10* and *pex5-10 ($PEX5_{454}$)* (Fig. 5-3A). This result suggests that the *pex5-10* PTS1 protein import defect is not altered by the $PEX5_{454}$ protein, as expected. To examine this rescue more directly *pex5-1 ($PEX5_{454}$)GFP PTS1, pex5-1 ($PEX5_{454}$)GFP PTS2, pex5-10 ($PEX5_{454}$)GFP PTS1* and *pex5-10 ($PEX5_{454}$)GFP PTS2* roots were examined. The results showed that PTS2 import was completely restored in *pex5-1 ($PEX5_{454}$)GFP PTS2* plants since GFP localization was similar to Col-0 *GFP PTS2* (Fig. 5-3B). PTS2 import was only partially restored in *pex5-10 ($PEX5_{454}$)GFP PTS2* plants while PTS1 protein import remained the same in *pex5-10 ($PEX5_{454}$)GFP PTS1* (Fig. 5-3B). Taken together these results suggest that the *$PEX5_{454}$* construct completely rescues PTS2 protein import in the *pex5-1* mutants, but only partially restores PTS2 import in *pex5-10*. The construct had no effect on PTS1 protein import.

A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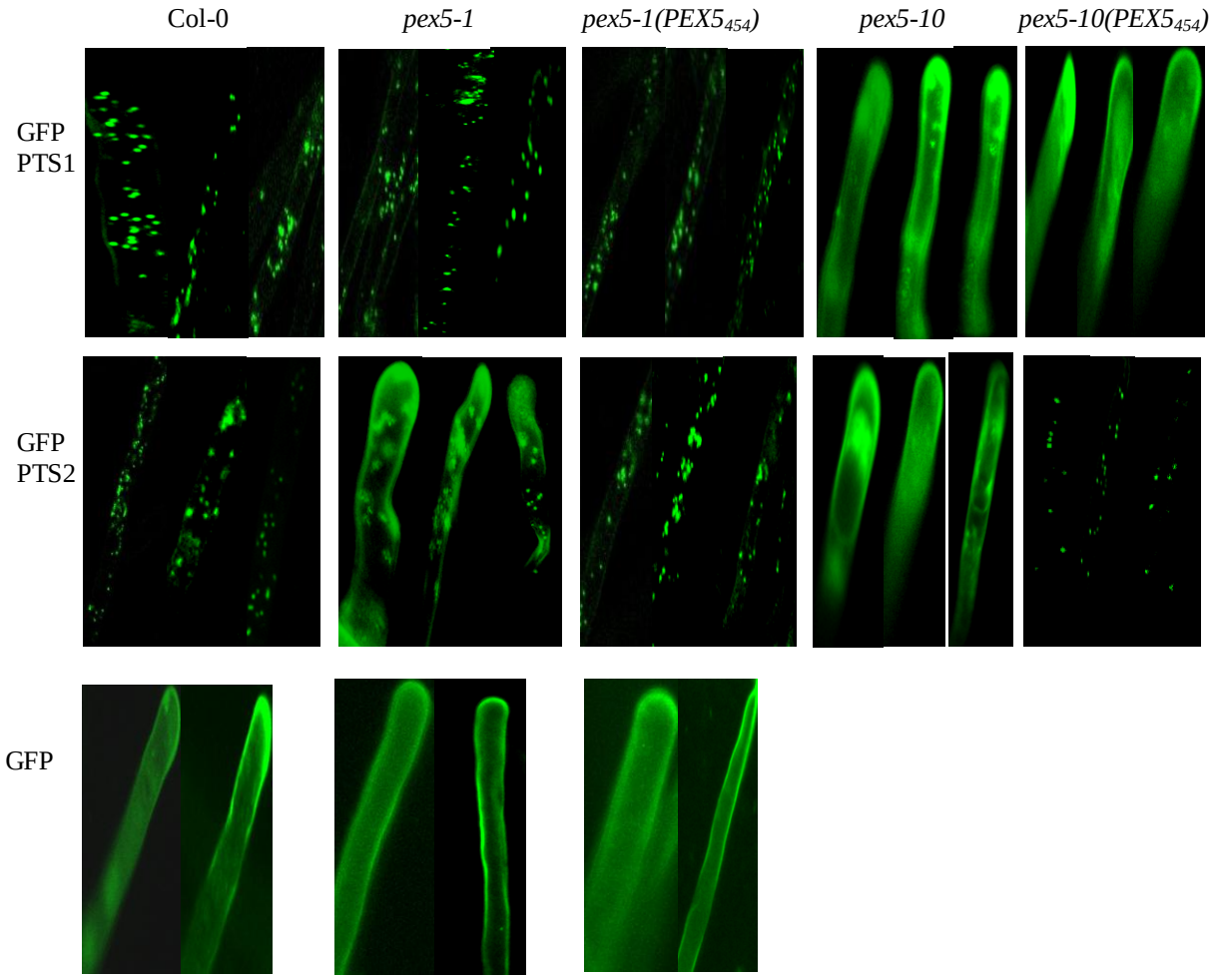
2
1.5
1
0.5
0
Wt
pex5-1
pex5-1 (PEX5454)
pex5-10
pex5-10 (PEX5454)
control
C12:0
nM H_2O_2/min
3.5
3
2.5
2
1.5
1
0.5
0
Wt
pex5-1
pex5-1 (PEX5454)
pex5-10
pex5-10 (PEX5454)
control

B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pex5-1(PEX5454)
pex5-10
pex5-10(PEX5454)
GFP PTS1
GFP PTS2
GFP

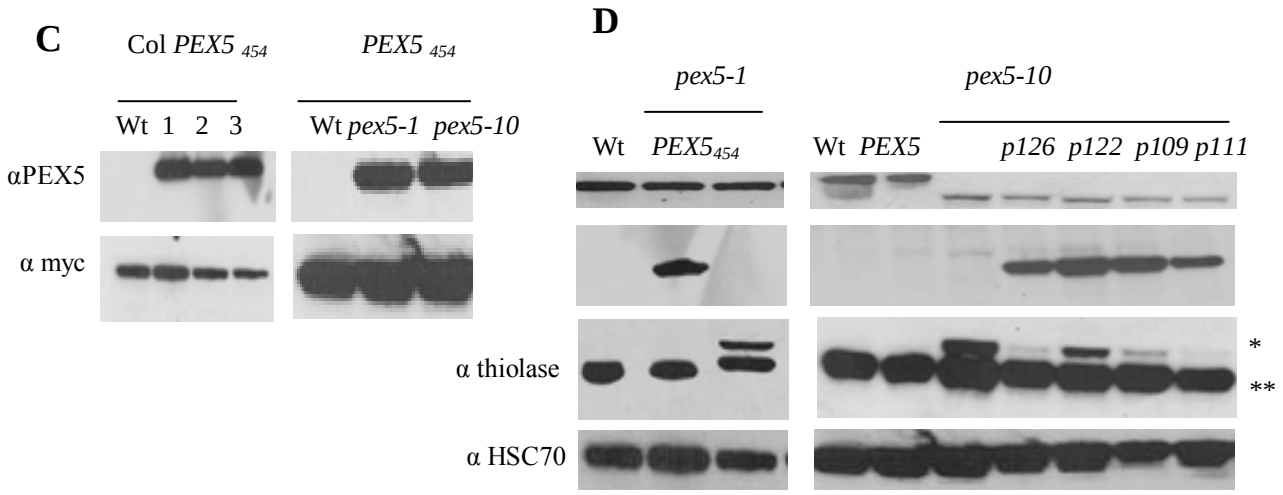


Figure 5-3. Peroxisomal defects in *pex5* mutant lines.

(A) Extracts from 4 d old Col-0 (Wt), *pex5-1*, *pex5-1 (PEX5$_{454}$)*, *pex5-10* and *pex5-10 (PEX5$_{454}$)* seedlings were tested for acyl-CoA oxidase activity with n-hexanoyl-CoA (C6:0) and lauroyl-CoA (C12:0). Data are presented as pmol H_2O_2 produced per minute. Error bars represent the standard error of the rates from three independent experiments. The control samples used water in place of plant extracts to account for background readings.

(B) GFP, GFP SKL and GFP PTS2 localization in Col, *pex5-1*, *pex5-10*, *pex5-1 (PEX5$_{454}$)* and *pex5-10 (PEX5$_{454}$)* roots.

(C) Total protein from wild-type and three independent transgenic Col-0 *(PEX5$_{454}$)* plants were analyzed by western blot for PEX5$_{454}$ accumulation. Total protein from homozygous progeny of *pex5-1* and *pex5-10* crossed to *Col (PEX5$_{454}$)* were analyzed by western blot for PEX5$_{454}$ accumulation.

(D) Western blot of total protein from wild-type, *pex5-10 (PEX5)*, *pex5-1*, *pex5-1 (PEX5$_{454}$)*, *pex5-10*, and *pex5-10 (PEX5$_{454}$)* plant lines p126, p122, p109, and p111 with PEX5, myc, and thiolase antibodies. PTS2 enzymes (thiolase) contain a targeting signal that is cleaved following import; protein size can indicate if the protein is localized in the peroxisome (mature short form, *) or if it remains in the cytoplasm (unprocessed long form, **).

For all blots, protein loading was determined by incubation with HSC70 antibody.

5.4. *pex5* mutant β-oxidation defects.

To examine the effect of losing PTS1 or PTS2 matrix protein import, I characterized the *pex5-1*, *pex5-10*, *pex5-1 ($PEX5_{454}$)*, and *pex5-10 ($PEX5_{454}$)* lines to look at growth, development, and peroxisomal function. The most striking phenotype for the *pex5-10* mutant was a severe germination defect. Typical germination rates for *pex5-10* lines ranged from 0 - 20%, which was seed line and harvest date dependent. In addition, any germination that occurred was delayed compared to wild type. To quantify this result, I plated progeny seeds from an F_2 segregating population of a *pex5-10* Col-0 backcross. After four days, no mutant plants had germinated, while I saw no defect in the heterozygous plants (Table 5-I), indicating the mutant segregated in a recessive fashion. If I nicked the seed coats of seeds that did not germinate, I saw a majority of seedlings developed. This population represented the homozygous mutant lines (Table 5-I).

As I was testing germination defects in *pex5-10*, I noticed a complete dependence of mutant seedling development on sucrose. This phenotype is consistent with a peroxisome-defective phenotype, as loss of fatty acid β-oxidation coincides with an inability to develop that can be rescued by application of sucrose (Hayashi et al., 1998). To determine the extent of fatty acid disruption in *pex5-10*, mutant seeds were plated on media without sucrose or on media supplemented with different concentrations of sucrose. By day 3, wild-type and *pex5-1* mutant seeds had germinated but none of the *pex5-10* seeds germinated (Table 5-2). Even when the *pex5-10* seed coats were nicked, hypocotyls would not elongate on media lacking sucrose. However, when *pex5-10* seed coats were nicked, hypocotyls could elongate on media supplemented with sucrose in a

concentration-dependent manner (Fig. 5-4A). The *pex5-1* mutant also displayed shorter hypocotyls than wild type on media without sucrose (Fig. 5-4A), as demonstrated previously (Zolman et al., 2000).

To determine whether the $PEX5_{454}$ protein rescued the fatty acid β-oxidation defects in *pex5-1* and *pex5-10,* I assayed the mutants for reduced sucrose dependence. The results showed that the $PEX5_{454}$ protein restored *pex5-1* growth without sucrose (Fig. 5-4B). Although *pex5-10* seeds did not germinate without nicking, seeds of *pex5-10 ($PEX5_{454}$)* lines could germinate and hypocotyls elongate in media supplemented with sucrose (Fig. 5-4B). When seed coats were nicked, the *pex5-10 ($PEX5_{454}$)* lines germinated on media with or without sucrose. Hypocotyl elongation in these lines was enhanced compared to *pex5-10*, although still greatly disrupted compared to wild type (Fig. 5-4C). Additionally, the *pex5-10 ($PEX5_{454}$)* seedlings established more robustly than the *pex5-10* seedlings (Fig. 5-4D).

To directly examine the degree of fatty acid β-oxidation, I quantified levels of the seed storage fatty acids eicosenoic acid (C20:1) in 6 day old seedlings. Wild-type seedlings show ~4% eicosenoic acid after 6 days (Fig. 5-4E). However, eicosenoic acid levels remained at ~25% in *pex5-10* seedlings and at 10% in *pex5-1* (Fig. 5-4E). These results indicate that the *pex5* mutants have reduced β-oxidation of seed-storage fatty acids, with the phenotypic strength paralleling the genetic defects. In contrast to *pex5-1,* levels were reduced similarly to wild type in *pex5-1 ($PEX5_{454}$)* seedlings (Fig. 5-4E). *pex5-10*

(PEX5$_{454}$) lines also showed reductions of eicosenoic acid to 23 – 17%, indicative of a somewhat increased rate of β-oxidation (Fig. 5-4E).

Taken together, these results indicate that the *pex5* mutants have disruptions in peroxisomal function that can be visualized by developmental defects. The PEX5$_{454}$ protein restored *pex5-1* fatty acid β-oxidation similar to wild-type levels. *pex5-1* has a PTS2 specific defect, so the complete rescue of this phenotype is consistent with the PEX5$_{454}$ protein increasing PTS2 import. The truncated protein only partially rescued the severe fatty acid β-oxidation defects in the *pex5-10* mutant, which has both PTS1 and PTS2 import defects. Although this rescue is only partial, the PEX5$_{454}$ addition does provide sufficient energy for the *pex5-10 (PEX5$_{454}$)* seeds to germinate unassisted.

Table 5-I: Germination responses (number) of 100 segregating *pex5-10* seeds.

Genotype	Parent #1	Parent #2	Parent #3	Parent #4
Wild-type	19	21	26	28
Heterozygous	52	54	52	49
Homozygous	0	0	0	0
After nicking:				
Wild-type	0	0	0	0
Heterozygous	0	0	0	0
Homozygous	24	25	20	23

Table 5-2. Unassisted germination percent of 100 seeds over time.

Seed lines	Day 2	Day 3	Day 4
Wt	7	100	100
pex5-10(PEX5)	9	100	100
pex5-1	10	100	100
pex5-1 ($PEX5_{454}$)	74	100	100
pex5-10	0	0	1
pex5-10 ($PEX5_{454}$) p111	0	38	100
pex5-10 ($PEX5_{454}$) p126	1	47	99
pex5-10 ($PEX5_{454}$) p109	8	50	100
pex5-10 ($PEX5_{454}$) p122	3	33	100

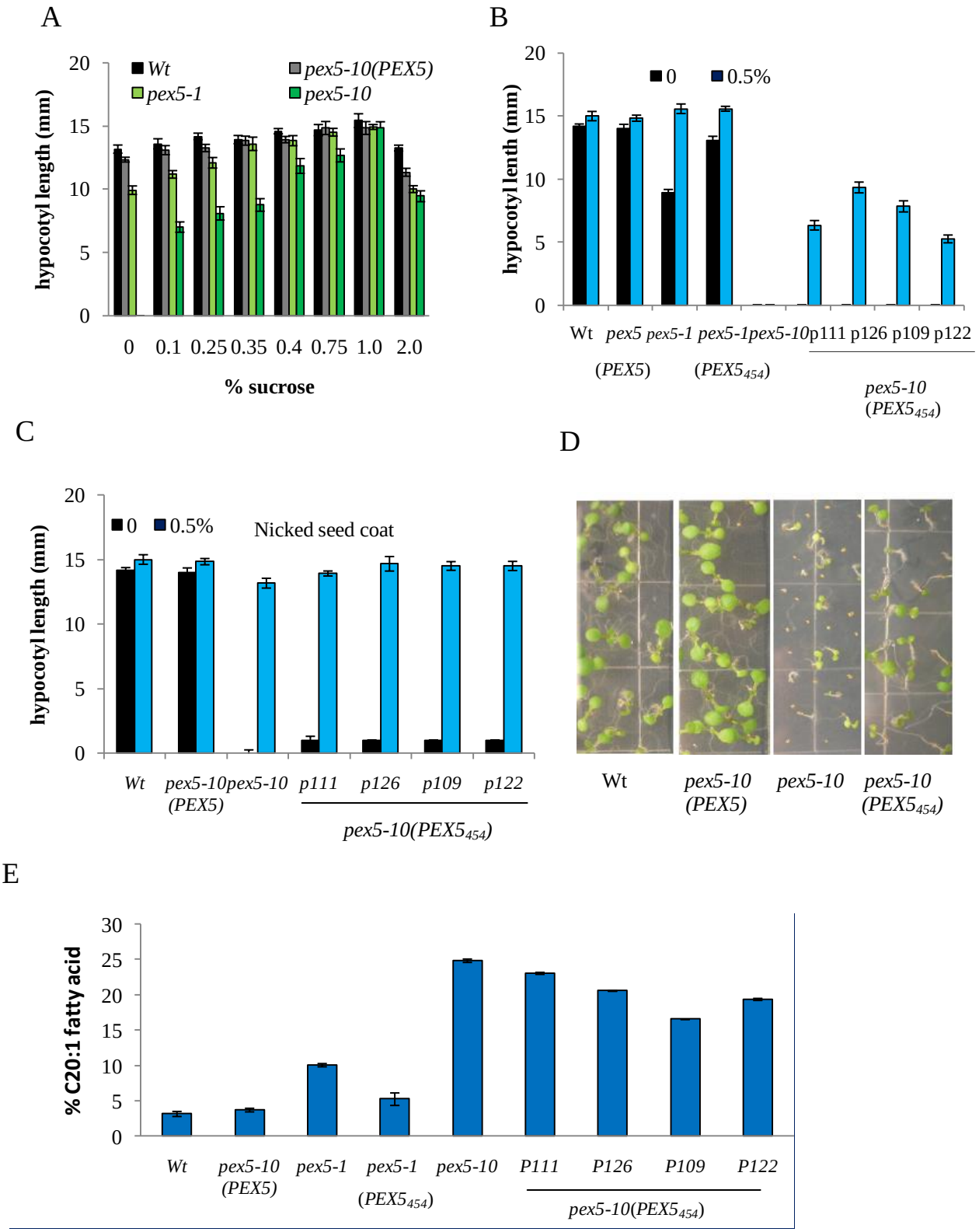

A
Wt
pex5-10(PEX5)
pex5-1
pex5-10
hypocotyl length (mm)
20
15
10
5
0
0 0.1 0.25 0.35 0.4 0.75 1.0 2.0
% sucrose
B
0
0.5%
hypocotyl lenth (mm)
Wt pex5 pex5-1 pex5-1pex5-10p111 p126 p109 p122
(PEX5) (PEX5454)
pex5-10
(PEX5454)
C
0
0.5%
Nicked seed coat
hypocotyl length (mm)
Wt pex5-10 pex5-10 p111 p126 p109 p122
(PEX5)
pex5-10(PEX5454)
D
Wt
pex5-10
(PEX5)
pex5-10
pex5-10
(PEX5454)
E
% C20:1 fatty acid
30
25
20
15
10
5
0
Wt pex5-10 pex5-1 pex5-1 pex5-10 P111 P126 P109 P122
(PEX5) (PEX5454)
pex5-10(PEX5454)

Figure 5-4. Fatty acid metabolism in *pex5* mutant seedlings.

(A) Hypocotyl length of seedlings grown 24 h in light and 6 d in dark on medium supplemented with increasing concentrations of sucrose. Error bars are the standard error of the means ($n \geq 12$).

(B) Hypocotyl length of seedlings grown 24 h in light and 6 d in dark on medium without sucrose or with 0.5% sucrose. Seed coats were not nicked. Error bars are the standard error of the means ($n \geq 12$).

(C) Hypocotyl length of seedlings grown 24 h in light and 6 d in dark on medium without sucrose or with 0.5% sucrose. *pex5-10* seed coats were nicked immediately after plating. Error bars are the standard error of the means ($n \geq 12$).

(D) Photographs depicting *pex5-10* plant lines. Plants were grown on complete media for 7 d. *pex5-10* seed coats were nicked after plating.

(E) Percent accumulation of C20:1 fatty acid in 6 d old seedlings grown in light on 0.5% sucrose medium. Error bars represent the standard error of the means of three biological replicates.

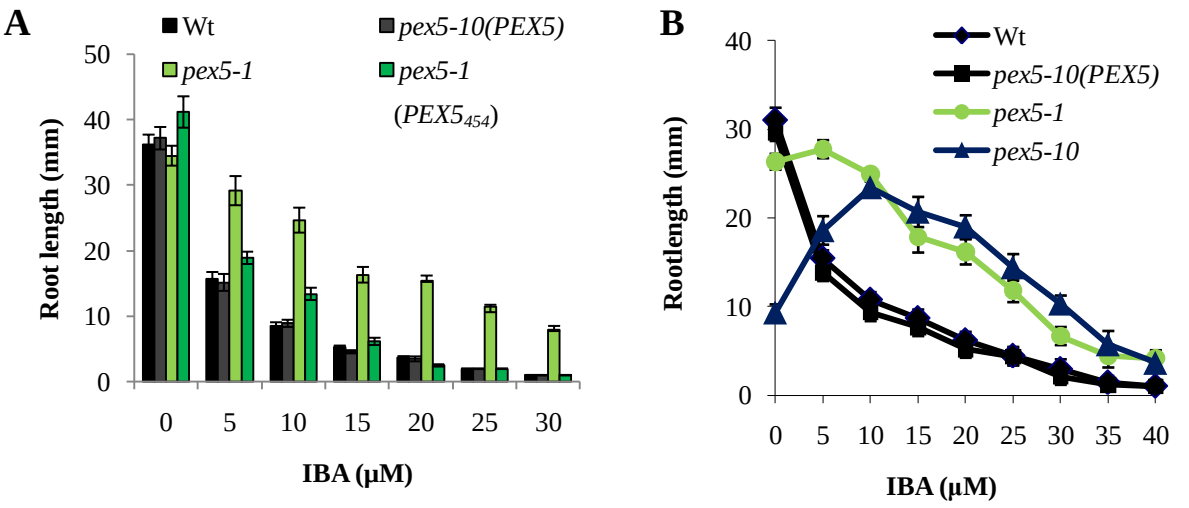
5.5. *pex5* mutant auxin responsiveness

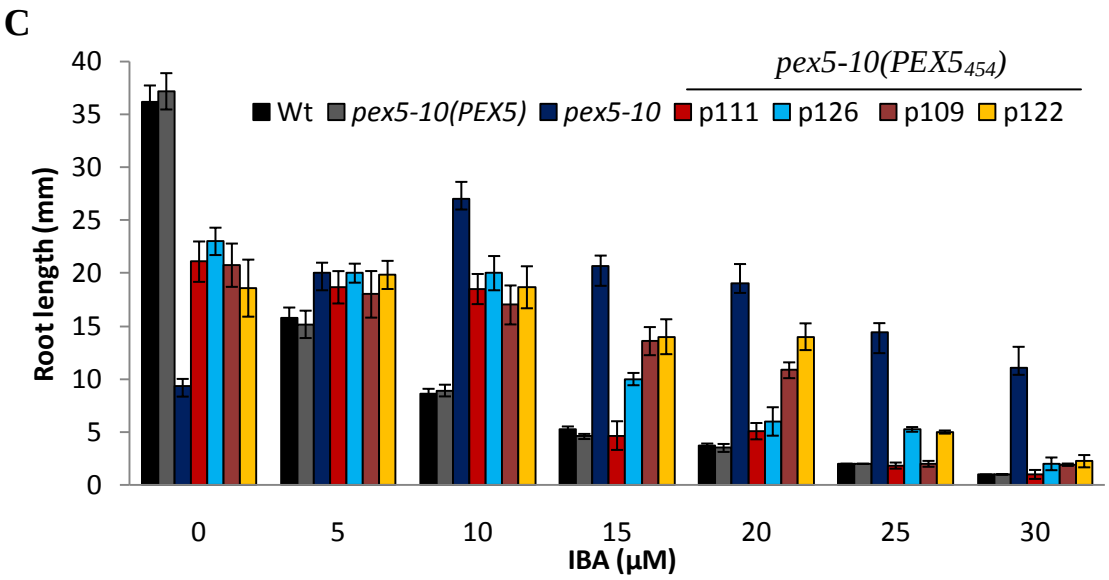
To investigate the extent of peroxisomal defects in the *pex5* mutants, I examined seedlings on IBA and 2,4-dichlorophenoxybutyric acid (2,4-DB), a synthetic IBA analog, which are metabolized in peroxisomes. Both *pex5-1* and *pex5-10* seedlings were insensitive to the inhibitory effects of IBA and 2,4-DB on root elongation (Fig. 5-5). *pex5-10* mutants have short roots on unsupplemented media but root length actually increases on 5 – 10μM IBA and remains longer than wild type over a range of IBA concentrations (Fig. 5-5B). Expression of $PEX5_{454}$ restored IBA sensitivity to *pex5-1* mutants (Fig. 5-5A). However, this truncated PEX5 protein only rescued the IBA insensitivity of *pex5-10* partially (Fig. 5-5C) and the degree of rescue varied among lines, ranging from the *pex5-10 (*$PEX5_{454}$*)* p111 with the highest degree of IBA resistance to p122 as the least IBA resistant (Fig. 5-5C). Alternatively, the *pex5-1 (*$PEX5_{454}$*)* and *pex5-10 (*$PEX5_{454}$) lines were all similarly sensitive to 2,4-DB (Fig. 5-5E).

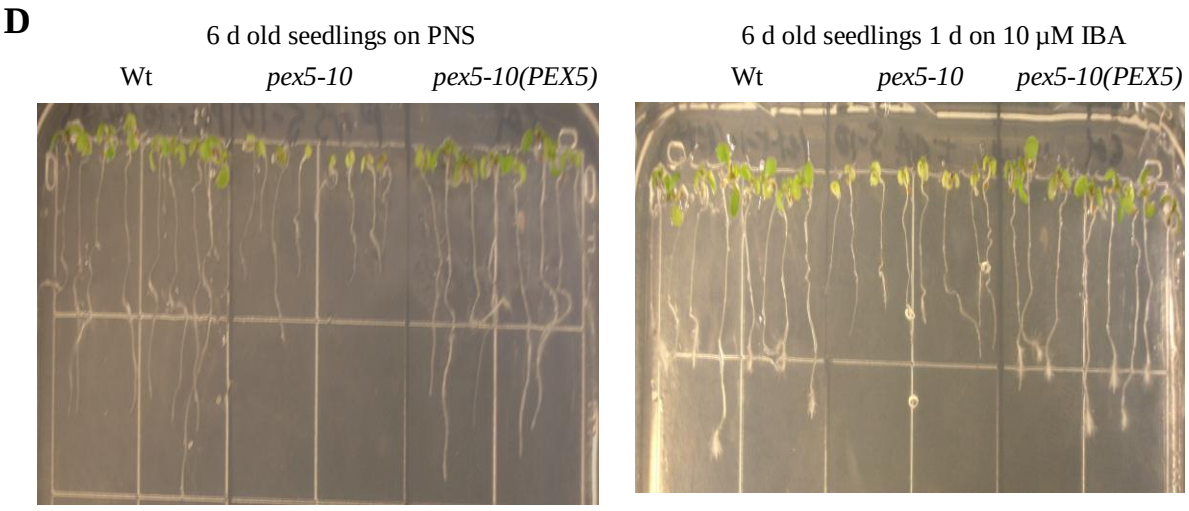
Since one role of IBA in plants is initiation of lateral roots, I examined mutants for lateral root formation. Both *pex5* mutants were compromised in lateral root formation on media without hormone or with IBA (Fig. 5-6). On media supplemented with IBA, the $PEX5_{454}$ protein rescued *pex5-1* seedling defects to wild-type levels, but lateral root formation was again only partially rescued in *pex5-10 (*$PEX5_{454}$*)* seedlings (Fig. 5-6A). In contrast, all *pex5* mutants initiated lateral roots similarly to wild type on NAA (Fig. 5-6A), indicating the defect is not in lateral root formation but in lateral root initiation. This defect can be visualized in *pex5-10 (*DR5-*GUS)* lines, which contain an auxin-inducible reporter (Guilfoyle, 1999) that highlights the lateral root primordia. *pex5-10* lines with this

transgene show reduced GUS levels and fewer lateral roots than Col-0 (DR5-*GUS*) lines (Fig. 5-6B).

The *pex5* mutants and the *PEX5*$_{454}$ lines responded normally to the synthetic auxins 2, 4-D and NAA (Fig. 5-7). However, as seen with IBA and 2,4-DB, *pex5-10* often shows increased growth on low concentrations of IAA (Fig. 5-7A), perhaps indicating that IAA levels are low in the mutant or that the exogenous application is increasing the ability of the mutant to grow and develop. Taken together, these data suggest that *pex5* mutants are compromised in the peroxisomal conversion of IBA β-oxidation to IAA.

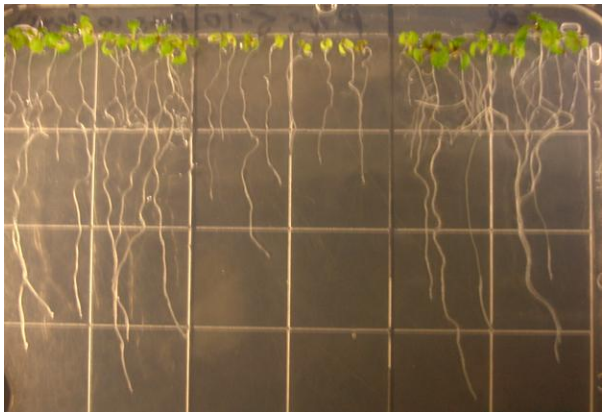

A
Wt
pex5-10(PEX5)
pex5-1
pex5-1
(PEX5454)
Root length (mm)
IBA (µM)
B
Wt
pex5-10(PEX5)
pex5-1
pex5-10
Rootlength (mm)
IBA (µM)


C
pex5-10(PEX5454)
Wt
pex5-10(PEX5)
pex5-10
p111
p126
p109
p122
Root length (mm)
IBA (µM)


D
6 d old seedlings on PNS
Wt
pex5-10
pex5-10(PEX5)
6 d old seedlings 1 d on 10 µM IBA
Wt
pex5-10
pex5-10(PEX5)

8 d old seedlings on PNS

Wt *pex5-10* *pex5-10(PEX5)*

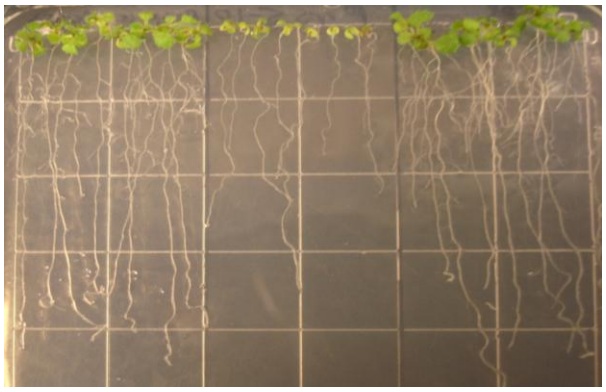


8 d old seedlings 5 d on PNS 3d on 10 µM IBA

Wt *pex5-10* *pex5-10(PEX5)*

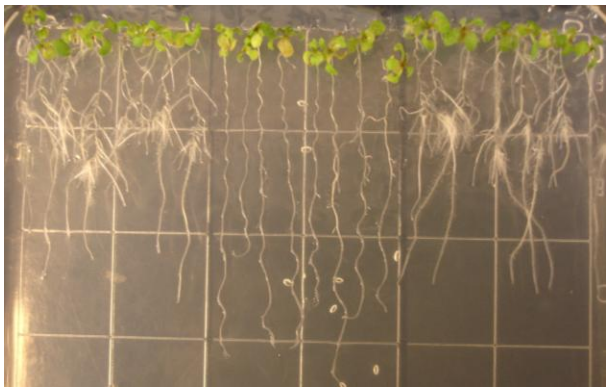
11 d old seedlings on PNS

Wt *pex5-10* *pex5-10(PEX5)*



11 d old seedlings 5 d on PNS, 6 d on 10 µM IBA

Wt *pex5-10* *pex5-10(PEX5)*



E

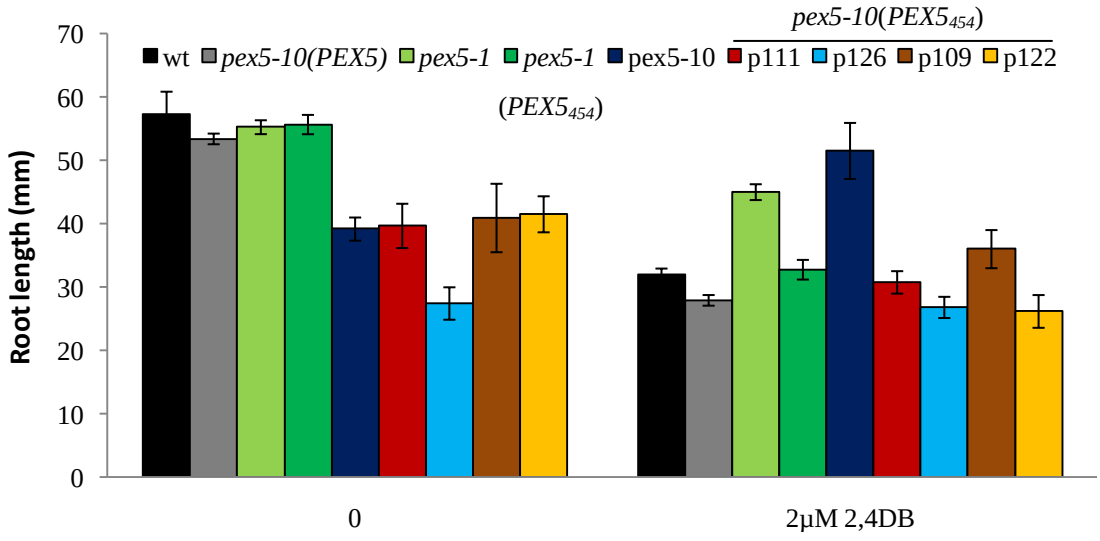


Figure 5-5. *pex5* mutant responses to IBA or the IBA analog 2,4-DB.

(A-C) Root length of 10 d old seedlings grown under continuous yellow light on 0.5% sucrose medium supplemented with the indicated concentration of IBA. Error bars represent the standard error of the means ($n \geq 12$).

(D) Photographs of 6, 8 and 11 d old wild-type, *pex5-10* and *pex5-10(PEX5)* seedlings grown on 0.5% sucrose or on 10 µM IBA.

(E) Root length of 10 d old seedlings grown under continuous light on 0.5% sucrose medium supplemented with the indicated concentration of 2,4-DB. Error bars represent the standard error of the means ($n \geq 12$).

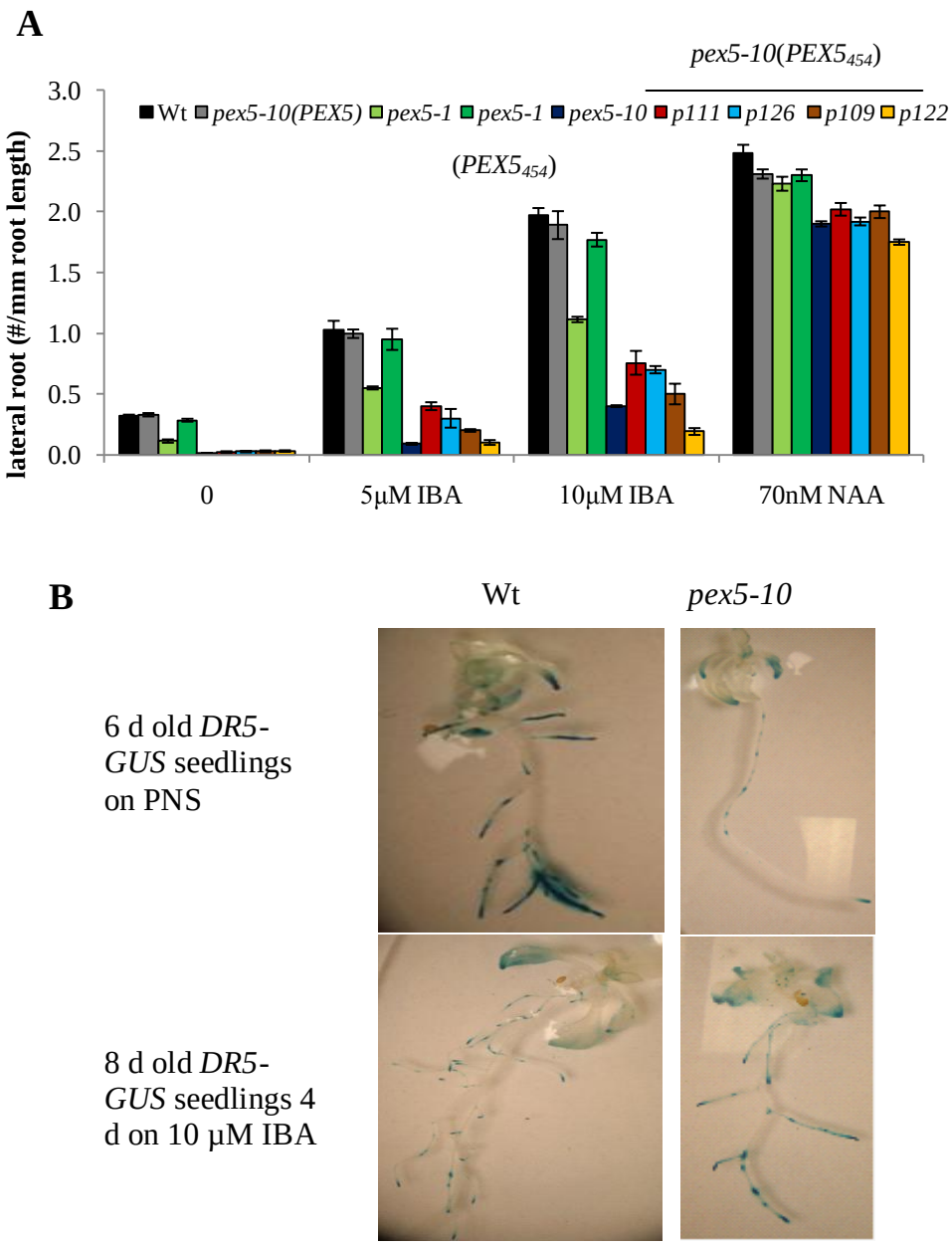


Figure 5-6. *pex5* lateral root formation.

(A) Lateral root density of seedlings grown on 0.5% sucrose medium for 5 d then transferred to medium with or without hormone for another 5 days. Data are shown as the number of lateral roots per mm root length. Error bars represent the standard error of the means ($n \geq 12$). (B) DR5-*GUS* expression in 8 d old Col-0 and *pex5-10* transgenic seedlings grown on complete media with 0.5% sucrose alone or with 10 µM IBA.

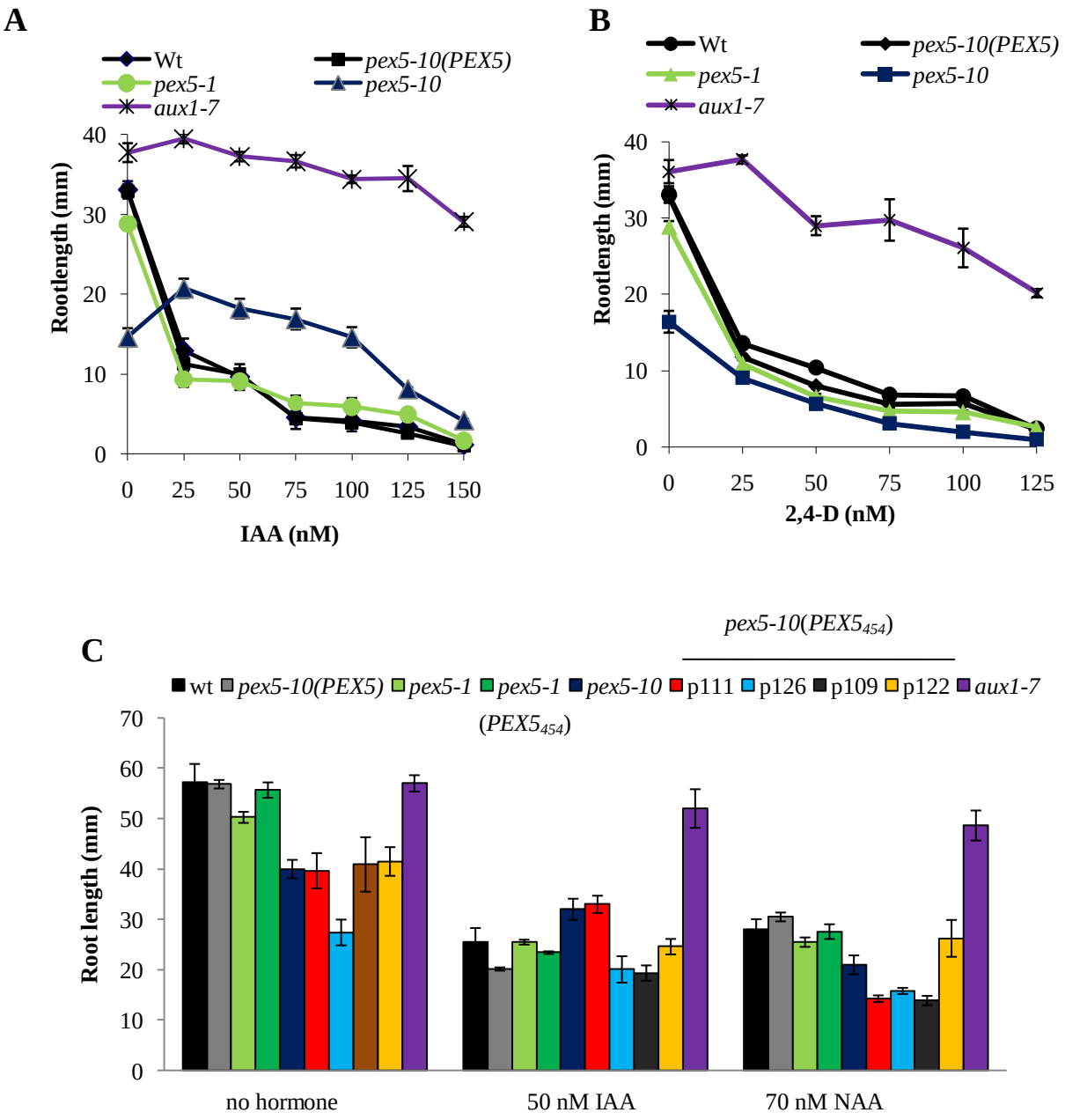


Figure 5-7. *pex5* mutant responses to IAA, 2,4-D and NAA.

Root length of 10 d old seedlings grown under continuous light on 0.5% sucrose medium supplemented with the indicated concentration of IAA (A, C), 2,4-D (B) and NAA (C). The auxin transport mutant *aux1* (Pickett et al., 1990) was used as a control to demonstrate insensitivity to IAA. Error bars represent the standard error of the means ($n \geq 12$).

5.6. *pex5-10* fertility and other peroxisomal defects

JA biosynthesis is initiated in the chloroplast, but 12-oxo-phytodienoic acid (OPDA) is transported to the peroxisome for β-oxidation to JA. JA biosynthesis and perception are required for male fertility (Wasternack and Kombrink, 2010). Measurement of siliques indicated that *pex5-10* makes fewer seeds than wild type (Fig. 5-8A). *pex5-1*silique lengths were normal. Further, I determined the average seed weight of 100 seeds and showed that *pex5-10* seeds weighed less than wild-type (Fig. 5-8B) and frequently are small and wrinkled. However, seed weight of *pex5-10 (PEX5$_{454}$)* was similar to wild type (Fig. 5-8B). These data suggest that *pex5-10* has minor fertility defects, based on defects in PTS2 import. I also examined the mutants for JA insensitivity by assessing root elongation on media supplemented with JA. None of the lines exhibited resistance to JA (Fig. 5-8C).

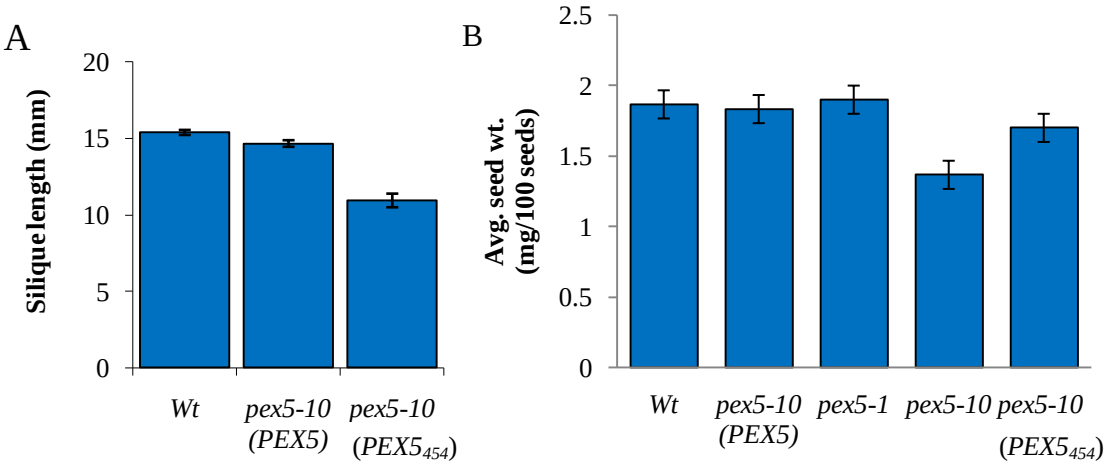
Valine catabolism is a peroxisomal process involving the CHY1 β-hydroxybutyryl-CoA hydrolase enzyme based on accumulation of a toxic intermediate that blocks β-oxidation (Zolman et al., 2001). Mutants defective in the *CHY1* gene exhibit sucrose dependence and IBA/2,4-DB insensitivity (Zolman et al., 2001; Lange et al., 2004; Lucas et al., 2007), similar to other peroxisomal β-oxidation defective mutants. In addition, the mutant lines were sensitive to root elongation inhibition by propionate and isobutyrate (Lucas et al., 2007). To examine if catabolism of these substrates also is compromised in the *pex5* mutants, I assayed wild-type and mutant seeds on propionate and isobutyrate. The *chy1* mutant (Zolman et al., 2001) was used as a control and exhibited a high degree of inhibition on propionate and isobutyrate media (Fig. 5-8D). In contrast, *pex5-1*

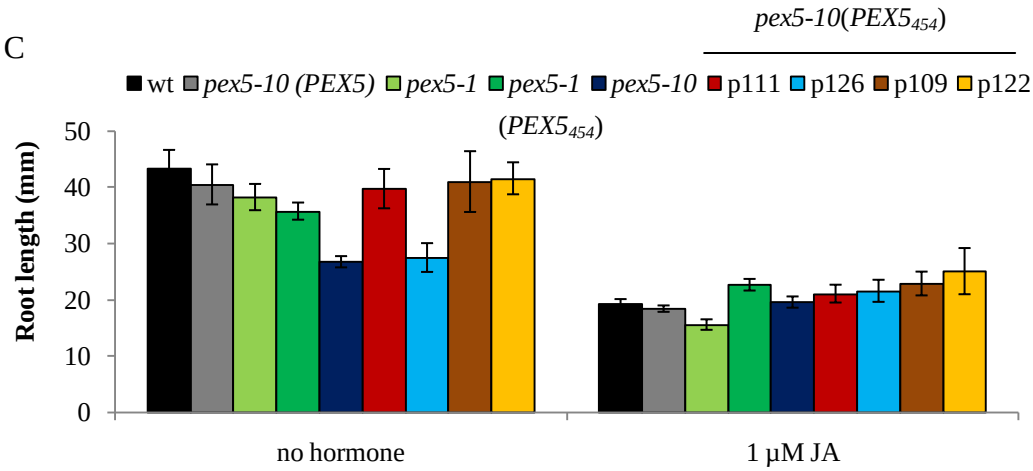
seedlings showed wild-type inhibition patterns on propionate and isobutyrate (Fig. 5-8D). The *pex5-10* seedlings showed a stronger inhibition on these compounds than wild type, although the defect was not as severe as *chy1* (Fig. 5-8D). Further, the $PEX5_{454}$ protein did not rescue this root elongation inhibition (Fig. 5-8D). This data suggests that these catabolism pathways are affected in the *pex5-10* mutant, and this defect may be based on disruptions in PTS1 protein import.

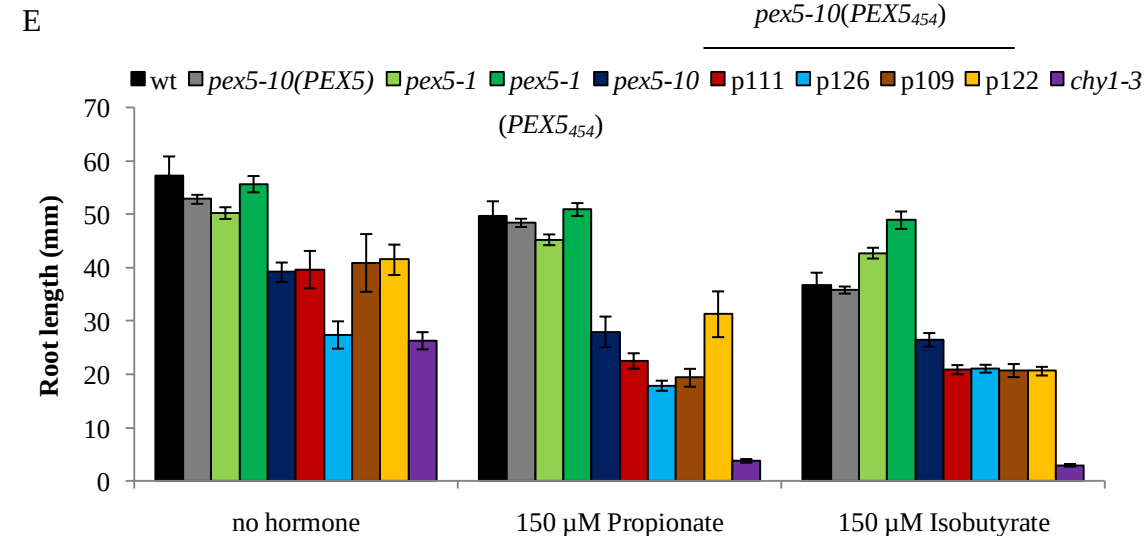
Leaf peroxisomes are involved in photorespiration to metabolize a non-useful photosynthetic side product, phosphoglycolate. Arabidopsis mutants defective in photorespiration demonstrate a pale green color due to reduced chlorophyll accumulation (Reumann, 2002). To determine if photorespiration is affected in the *pex5* mutants, I quantified total chlorophyll in seedlings and adult leaves. *pex5-10* seedlings had a pale green color (Fig. 5-5D) and showed a reduction in total chlorophyll compared to wild type (Fig. 5-8E). This chlorophyll defect was more severe than in the *pex6-1* mutant, a mutant defective in PEX6 (Zolman and Bartel, 2004), and the $PEX5_{454}$ protein did not rescue this defect in seedlings (Fig. 5-8E). Total chlorophyll of *pex5-1* seedlings was similar to wild type. Interestingly, total chlorophyll of both *pex5-10* and *pex5-10* $PEX5_{454}$ adult leaves were similar to wild type, while the *pex6-1* plants still maintained this defect (Fig. 5-8E).

Further, peroxisomes house many metabolic processes that produce H_2O_2. Peroxisomes are adapted to detoxifying these compounds before they accumulate to damaging levels. To test the ability of the detoxifying enzymes to enter peroxisomes, I stained for levels of

H_2O_2 using DAB (Thordal-Christensen et al., 1997). Wild-type and mutant leaves were stained with DAB to determine H_2O_2 accumulation. Both *pex5-10* and *pex5-10 (PEX5$_{454}$)* leaves accumulated a brown precipitate indicative of H_2O_2 accumulation (Fig. 5-8F). This result indicates that H_2O_2 is not efficiently inactivated in *pex5-10*. The PEX5$_{454}$ protein only partially rescues the H_2O_2 inactivation defect in *pex5-10* (Fig. 5-8F).


A
Silique length (mm)
20
15
10
5
0
Wt
pex5-10 (PEX5)
pex5-10 (PEX5454)
B
Avg. seed wt. (mg/100 seeds)
2.5
2
1.5
1
0.5
0
Wt
pex5-10 (PEX5)
pex5-1
pex5-10
pex5-10 (PEX5454)


C
pex5-10(PEX5454)
wt
pex5-10 (PEX5)
pex5-1
pex5-1
pex5-10
p111
p126
p109
p122
(PEX5454)
Root length (mm)
50
40
30
20
10
0
no hormone
1 µM JA


E
pex5-10(PEX5454)
wt
pex5-10(PEX5)
pex5-1
pex5-1
pex5-10
p111
p126
p109
p122
chy1-3
(PEX5454)
Root length (mm)
70
60
50
40
30
20
10
0
no hormone
150 µM Propionate
150 µM Isobutyrate

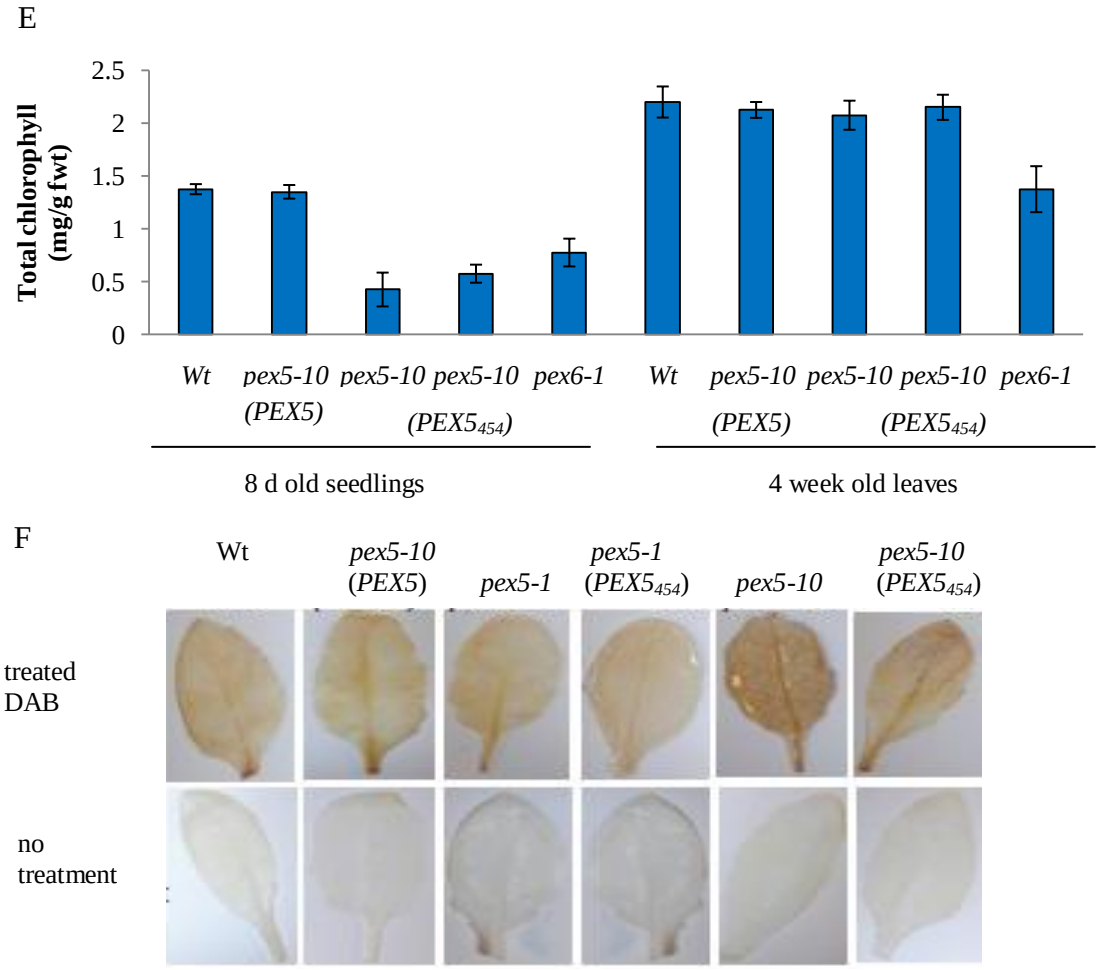


Figure 5-8. *pex5-10* fertility, photorespiration and H_2O_2 inactivation defects.
(A) Length of 5th silique on the main stem. Error bars represent the standard error of the means ($n \geq 18$). (B) Average seed weight in mg per 100 seeds. Error bars represent the standard error of the means ($n \geq 4$). (C) Root length of 10 d old on 0.5% sucrose medium supplemented with 1 µM JA. Error bars represent the standard error of the means ($n \geq 12$). (D) Root length of 10 d old seedlings grown on 0.5% sucrose medium supplemented with the indicated concentrations of propionate and isobutyrate. Error bars represent the standard error of the means ($n \geq 12$). (E) Total chlorophyll content in mg per g fresh weight for 8 d old seedlings grown and 4 wk old leaves of plants grown in soil with normal watering. Error bars represent the standard error of the means ($n \geq 6$).
(F) 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining showing accumulation of H_2O_2 in 2 wk old wild-type, *pex5-10(PEX5)*, *pex5-1*, *pex5-1(*PEX5$_{454}$*)*, *pex5-10* and *pex5-10(*PEX5$_{454}$*)* leaves.

5.7. *pex5-10* adult development

I analyzed plant growth and development to demonstrate how *pex5-10* is able to progress. I found initial issues with growth, as evidenced by short root elongation and small aerial development. Once the plants had begun photosynthetic growth, they seemed to recover to near wild-type growth. *pex5-10* plants showed reduced rosette diameter (Fig. 5-9A). However, overall plant architecture remained fairly normal, with the number of stems produced, plant height, and date of bolting patterns matching wild type (Fig. 5-9).

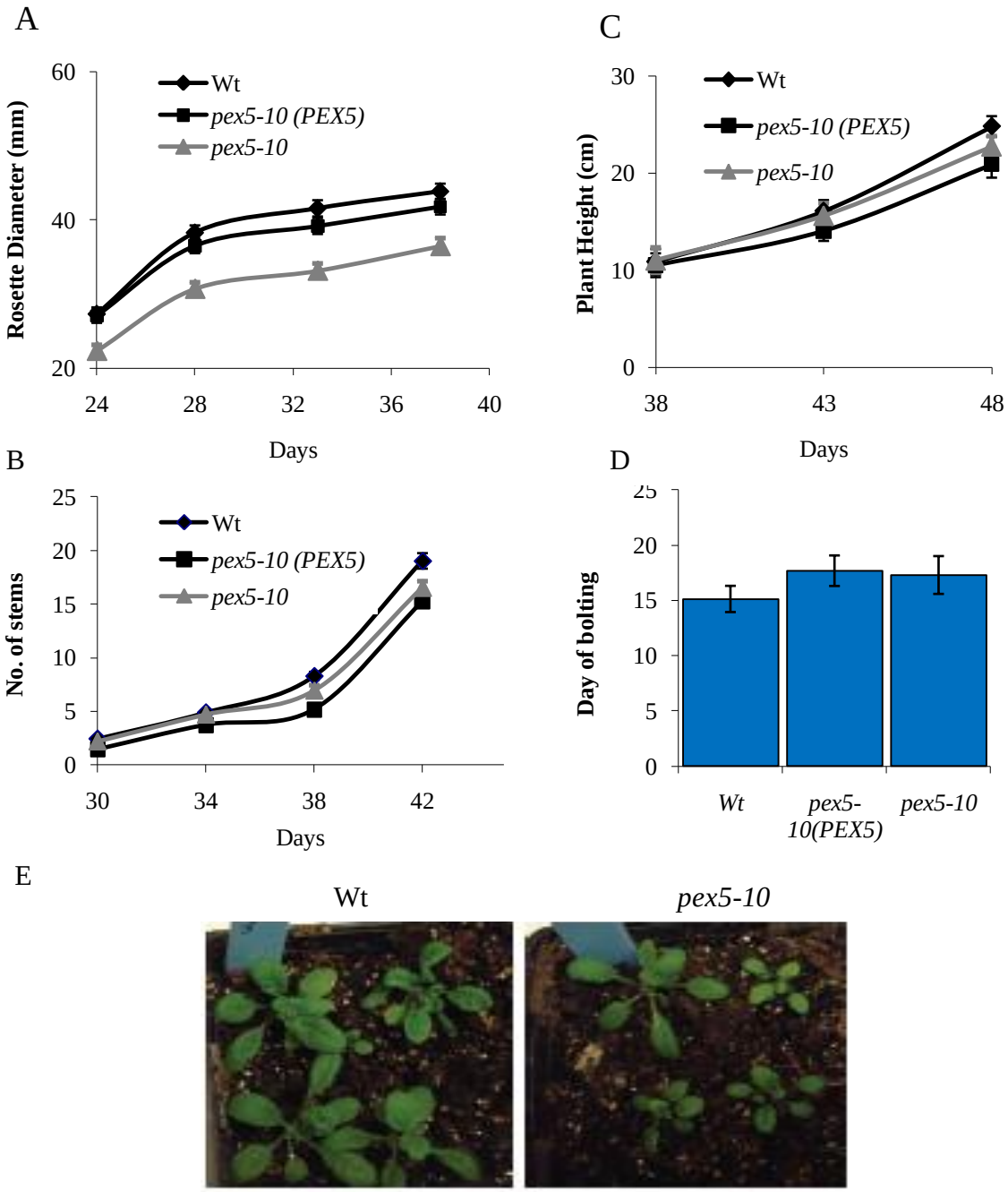


Figure 5-9. *pex5-10* growth and development

Wt, *pex5-10(PEX5)* and *pex5-10* plants were grown under continuous white light at 22^{0}C with normal watering two times per week. Plants were measured for (A) Rosette diameter oat 24, 28, 32, 36 and 40 d days, (B) the number of stems produced at 30, 34, 38 and 42 d were counted, (C) plant height were measured at 38, 43 and 48 d days, (D) number of day seedlings started to bolt after they were transferred to soil. Error bars represent the standard error of the means ($n \geq 18$). (E) Photographs of 3 weeks old soil grown Wt and *pex5-10* plants.

5.8. Discussion

pex5-10 is a loss-of-function mutant resulting from a T-DNA insertion in the *PEX5* gene. The isolation of this mutant was unexpected; the original hypothesis was that a *pex5* insertion mutant would be embryo lethal, similar to several other peroxisome-defective mutants (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005). When I analyzed the effect of the insertion at the genetic and protein level, however, I found the insertion effects were atypical. The insertion does lead to a loss of gene expression, as seen using primers in the exon in which the insertion is located (Fig. 5-2D). However, at some frequency, this exon and the insertion are spliced out in the mutant (Fig. 5-2D). This excision results in a protein with a deletion (Fig. 5-2B, C); the mutant protein is missing four of the first five PPRs of the protein (Fig. 5-1A). This splicing event happens incompletely, as the mutant protein band is of lower intensity than the wild-type protein (Fig. 5-2B). A global analysis of the effects of T-DNA insertions located in exons revealed that only 2% of those studied resulted in truncated proteins being formed, compared to 88% of the samples resulting in loss of protein accumulation (Wang, 2008).

Isolation of this splicing event in the insertion mutant is fortuitous, as it allows study of PEX5 function when a true *pex5* null likely would be embryo lethal. The mutant pex5-10 protein must retain just enough activity to maintain the mutant plant growth and development, particularly in adult plants. The defects in *pex5-10* are stronger than *pex5-1*, but fall in a similar spectrum. This allelic spectrum allows me to draw conclusions about the function of PEX5 in plant growth and development. PEX5 is clearly required

for import of peroxisome matrix proteins, as loss of PEX5 causes mislocalization of thiolase (Zolman et al., 2005; Lingard and Bartel, 2009; Ramón and Bartel, 2010), catalase (Lingard and Bartel, 2009; Lingard et al., 2009), isocitrate lyase (Lingard et al., 2009), malate synthase (Lingard and Bartel, 2009), and peroxisomal malate dehydrogenase (Lingard and Bartel, 2009; Ramón and Bartel, 2010). However, the mutant is able to correctly localize the peroxisomal membrane protein PEX14 (Lingard and Bartel, 2009), indicating peroxisomal structures are intact and that membrane protein import occurs normally. This loss of matrix import can be seen in reduced peroxisomal matrix protein enzymatic activity and GFP localization (Fig. 5-3A, B). Therefore, this mutant has enabled me to nicely score the effects of loss of peroxisomal function, as the mutant strongly disrupts all peroxisome matrix processes by not importing the required proteins.

Despite the numerous functions of peroxisomes throughout a plant life cycle (Kaur, 2009), perhaps PEX5 is most critical during early development when peroxisomal fatty acid β-oxidation is required for germination. The *pex5-10* mutant has a low degree of germination (0-20%) without assistance (Table 5-1, 5-2) and a strict requirement for exogenous sucrose during seedling development (Fig. 19; (Lee et al., 2006; Lee, 2007; Lingard and Bartel, 2009; Ramón and Bartel, 2010). Despite severe germination and seedling developmental phenotypes, some mutants survive and develop into an adult plant. Seedlings also are unable to metabolize IBA/2,4-DB, resulting in alterations in secondary root development (Fig. 5-5, 5-6; (Lee et al., 2006; Lee, 2007; Lingard and Bartel, 2009; Ramón and Bartel, 2010). As plants develop and start photosynthesis, PEX5

accumulation decreases (Fig. 5-1B). This trend explains the strong seedling defects in *pex5-10* seedlings, but relatively normal phenotype of the adult plants (Fig. 5-9). Other peroxisomal processes, including photorespiration and JA synthesis, also are affected in the mutant. *pex5-10* plants show reduced fertility (Fig. 5-8) indicating the importance of retaining PEX5 function in adult plants as peroxisomal JA synthesis pathway is essential for male fertility (Wasternack and Kombrink, 2010). The mutant also accumulates H_2O_2 (Fig. 5-8E), likely resulting in damage. The enzymatic studies of peroxisomal processes (Fig. 5-3A) and examination of β-oxidation using eicosenoic acid as a marker and GFP localization (Fig. 5-3B) demonstrate the effects of decreased peroxisomal import in *pex5-10* and explain the above defects in plant development. In addition to studies with the *pex5-10* insertion mutant, Hayashi et al. (2005) used RNAi to decrease *PEX5* levels, resulting in a dwarf phenotype and photorespiration defects. Similar to *pex5-10*, the mutants were sucrose dependent and resistant to 2,4-DB and showed defects in both PTS1 and PTS2 import (Hayashi et al., 2005). As expected, the mutant is strongly defective in a number of peroxisomal processes, with resulting defects in plant growth and development. Each of these defective processes reveals the importance of PEX5 in peroxisomal function and the various roles of peroxisomes in plant growth and development.

Peroxisomal import relies on two major import pathways. PEX5 directly imports PTS1 proteins, but also is required for PTS2 import (Hayashi et al., 2005; Woodward and Bartel, 2005). The *pex5-10* mutant shows defects in both PTS1 and PTS2 import. The *pex5-1* mutant disrupts only PTS2 import (Woodward and Bartel, 2005) because of a Ser-

to-Leu point mutation in the PEX7 binding domain (Zolman et al., 2000). Therefore, although both mutants allow us to study defects in peroxisomal matrix protein import, neither is appropriate to specifically study PTS1 import. I wanted to generate a line defective specifically in PTS1 import so I could study the consequences of disruptions in PTS1 and PTS2 import separately. The *pex5-10* (*$PEX5_{454}$)* construct expresses the N-terminal region of *PEX5*, including the PPR domains required for binding to peroxisomal membrane proteins and the PEX7 binding domain. These domains should allow normal peroxisomal import for the PEX7-bound PTS2 proteins via PEX5. However, $PEX5_{454}$ is missing the tetratricopeptide domains required for binding the tripeptide PTS1 signal at the C-terminus of the protein. I crossed this construct into the *pex5-1* and *pex5-10* mutants, which each retain some activity. I hypothesized that $PEX5_{454}$ would partially complement each mutant. Although *pex5-1* and *pex5-10* retain much of thiolase (a PTS2 model protein) in the unprocessed, cytosolic form, the transgenic lines exhibit increased peroxisomal import (Fig. 5-3C). In addition, $PEX5_{454}$ rescued import of the peroxisomally-localized ACX enzymes. ACX4 has a PTS1 signal and is known to be the primary enzyme required for oxidation of short-chain fatty acid substrates (Hayashi et al., 1999). The *pex5* mutants cannot oxidize C6:0 substrates, a defect which is not affected by insertion of the *$PEX5_{454}$* construct (Fig. 5-3A). ACX3 is a major enzyme affecting C12:0 oxidation; this enzyme has a PTS2 targeting signal (Eastmond et al., 2000; Froman et al., 2000). ACX3 activity is reduced in both mutants, but is recovered partially in the transgenic line (Fig. 5-3A), indicating that the $PEX5_{454}$ protein is increasing PTS2 import. GFP localization studies also showed that *$PEX5_{454}$* rescued PTS2 import in *pex5-*

1 and *pex5-10*. Therefore, the defects in *pex5-10 (PEX5$_{454}$)* result from a specific defect in PTS1 import.

Interestingly, comparisons of the *pex5* and *pex5 (PEX5$_{454}$)* lines have allowed me to separate which import pathways are required for several peroxisomal processes. Comparison of the *pex5-1* mutant and the *pex5-1 (PEX5$_{454}$)* lines reveals a fairly complete phenotypic rescue under all conditions, consistent with the idea that *pex5-1* defect primarily disrupts PTS2 import and that PEX5$_{454}$ restores PTS2 activity. The PEX5$_{454}$-mediated recovery of PTS2 activity is evident with increased seed storage fatty acid utilization (Fig. 5-4E), increased development in the absence of sucrose (Fig. 5-4B), and responses to IBA (Fig. 5-5A, 5-6A). Similarly, the *pex5-10 (PEX5$_{454}$)* plants show improved growth and development, although the transgenic lines remain impaired. Expression of *PEX5$_{454}$* in *pex5-10* promotes increased germination without physical stimuli compared to *pex5-10*, although substantial delays remain compared to wild type (Table 5-1). Improvements also include an increased ability to grow without sucrose supplementation (Fig. 5-4B-D), higher β-oxidation rates (Fig. 5-4E), greater responses to IBA stimuli (Fig. 5-5C, 5-6A), and improved ability to make seeds (Fig. 5-8B).

pex5-10 (PEX5$_{454}$) plants show only a partial rescue of the *pex5-10* phenotypes, consistent with the idea that PTS1 import still is disrupted in these lines. Although the improved import of PTS2-containing proteins is assisting in peroxisomal processes, most pathways require enzymes with both PTS1 and PTS2 signals. Interestingly, three assays did not show any changes between the mutant parent and the transgenic line. First, an

inability of plants to metabolize both propionate and isobutyrate results in a hypersensitive phenotype, likely due to the accumulation of toxic intermediates in the pathway that inhibit growth (Lucas et al., 2007). *pex5-10* is sensitive to both of these compounds, which is not affected by $PEX5_{454}$ expression (Fig. 5-8C). This result suggests that all of the enzymes (or at least the key rate-limiting enzymes) involved in processing these metabolites have PTS1 import signals. This mechanism is consistent with the suggested enzymatic pathways (Lucas et al., 2007). Also, photorespiration rates were essentially the same in *pex5-10* and *pex5-10 (*$PEX5_{454}$*)* lines (Fig. 5-8D). The fact that these defects are not affected by $PEX5_{454}$ expression, and therefore may specifically require PTS1 import, is consistent with the result that the *pex5-1* does not exhibit a defective phenotype in any of these assays. In contrast, responses to 2,4-DB (Fig. 5-5E) were completely rescued, revealing that the disruption in *pex5-10* may be caused by mislocalization of a key PTS2-containing protein that can be imported by $PEX5_{454}$. Recent results indicate that 2,4-DB β-oxidation to 2,4-D may occur using a distinct and/or overlapping set of enzymes as IBA β-oxidation to IAA, although the only protein with known differential activity has a PTS1 signal (Wiszniewski et al., 2009).

In this study, I determined the effect of the *pex5-10* mutation on *PEX5* gene expression and protein accumulation. I then examined the importance and function of PEX5 in peroxisomal targeting by observing the phenotypes of *pex5* mutants. The defects shown here confirm the critical role of PEX5 in peroxisomal metabolism. The studies further reveal the role of peroxisomal metabolism in many different aspects of plant growth and development, primarily in germination and early seedling development. Additional

comparisons of the three *pex5* lines will be useful for enhanced studies of the protein complements of plant peroxisomes and for studies of the mechanism of plant peroxisomal matrix protein import.

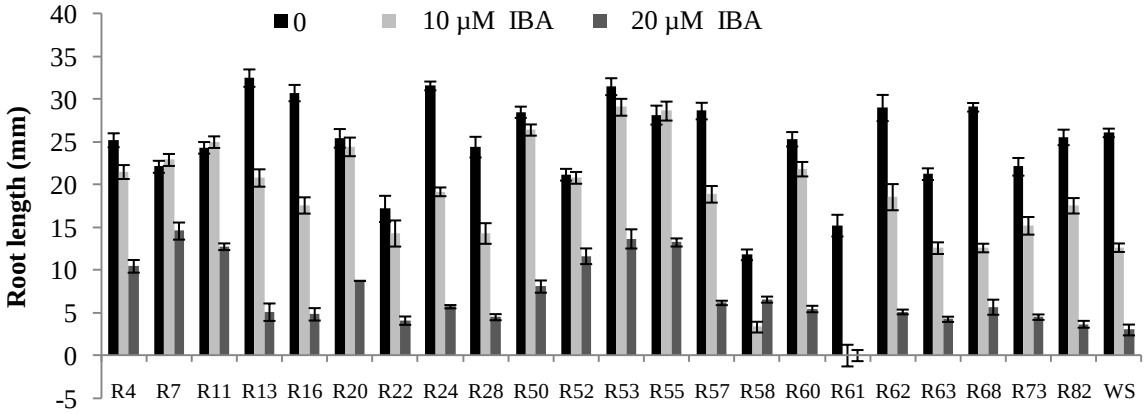
CHAPTER 6: Screening and isolation of additional peroxisome mutants in Arabidopsis Wassilewskija (Ws) accession.

6.1. Ethylmethanesulfonate (EMS) mutagenized seeds

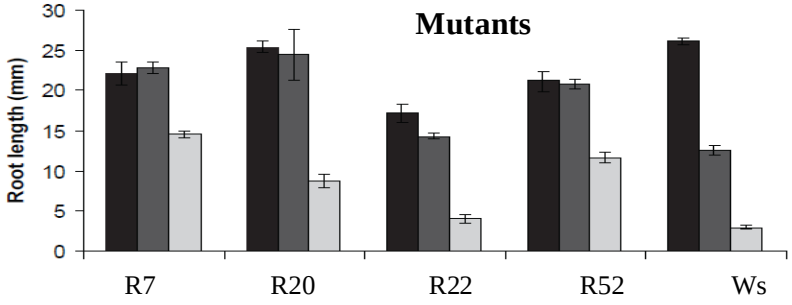
Ethylmethanesulfonate (EMS) mutagenized Ws seeds were purchased from Lehle seeds. Seeds were screened for IBA resistance on 15µM IBA. Long roots on IBA are indicative of a peroxisome defect. This defect can either be in peroxisome import, recycling, or IBA-β-oxidation. Thus, seedlings with long roots were transferred to soil and M_3 seeds were collected. M_3 seeds were re-screened on IBA to confirm IBA resistant phenotypes. Mutants were screened for IAA sensitivity on increasing IAA concentrations. This screen was done to rule out IAA (*aux*) mutants in the mutant population since the focus of the lab is to elucidate proteins involved specifically in peroxisome processes. For the lateral root initiation assay, mutants were plated on PNS and allowed to develop for 4 days after which they were transferred to media containing 10µM IBA for 5 days. After 5 days on hormone, the number of lateral roots was counted. A defect in the number of lateral roots produced by a seedling is also an indication of a block in a peroxisome process. Auxins initiate lateral root formation, so if IBA-β-oxidation is blocked the seedling will produce fewer lateral roots. Further, dependence of mutants on sucrose for germination and seedling establishment was determined. The dependence of mutants on sucrose for germination and establishment is an indication of a block in fatty acid β-oxidation, a peroxisome process in plants. The mutants isolated from this screen were designated R or N. The phenotypes of the R and N mutants are shown in (Fig. 6-1; Table 6-1).

8 days

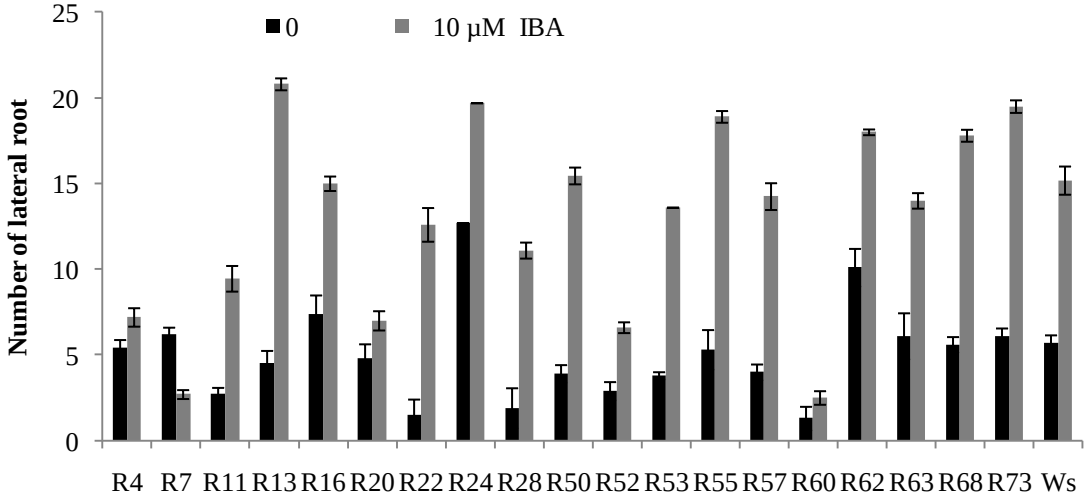
A

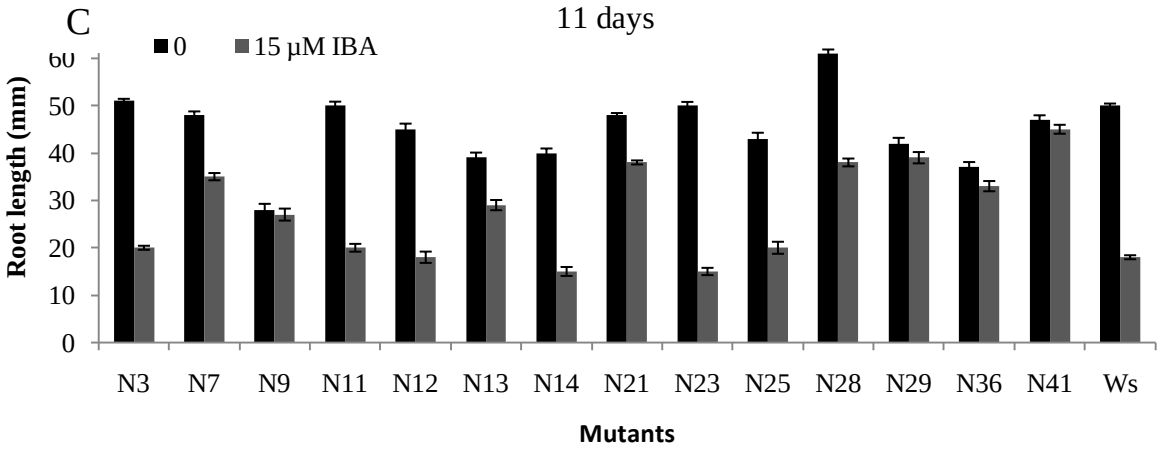


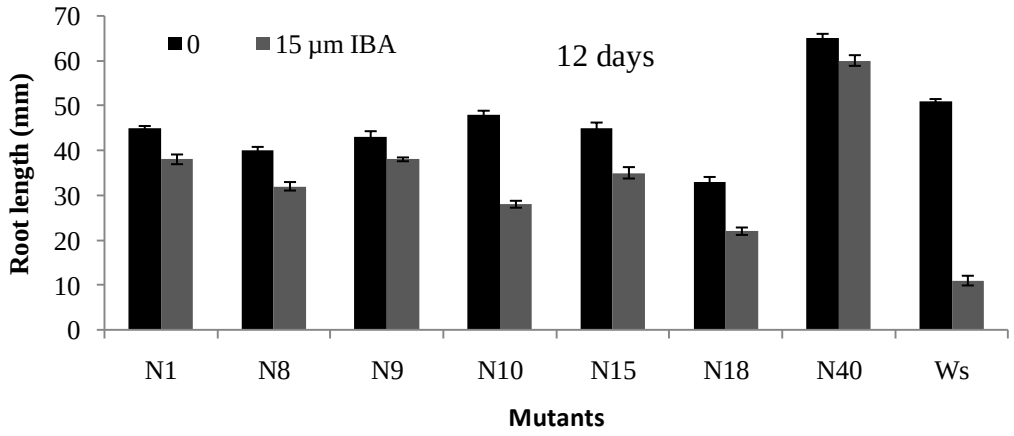
8 days

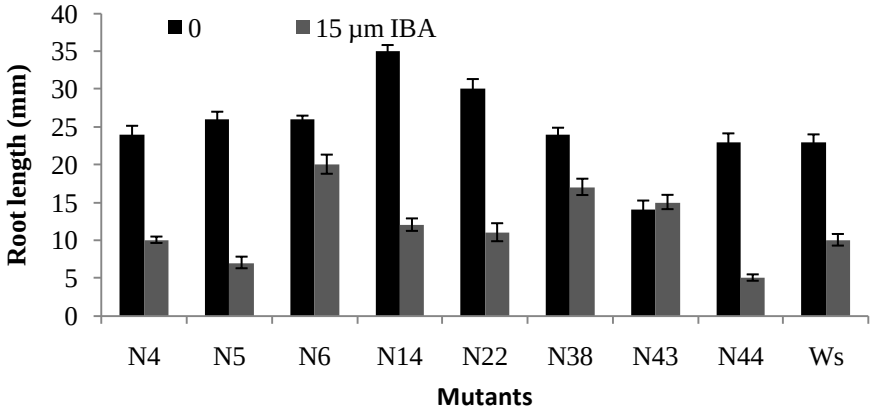


B




C
11 days
0
15 µM IBA
Root length (mm)
60
50
40
30
20
10
0
N3
N7
N9
N11
N12
N13
N14
N21
N23
N25
N28
N29
N36
N41
Ws
Mutants


0
15 µm IBA
12 days
Root length (mm)
70
60
50
40
30
20
10
0
N1
N8
N9
N10
N15
N18
N40
Ws
Mutants


0
15 µm IBA
Root length (mm)
40
35
30
25
20
15
10
5
0
N4
N5
N6
N14
N22
N38
N43
N44
Ws
Mutants

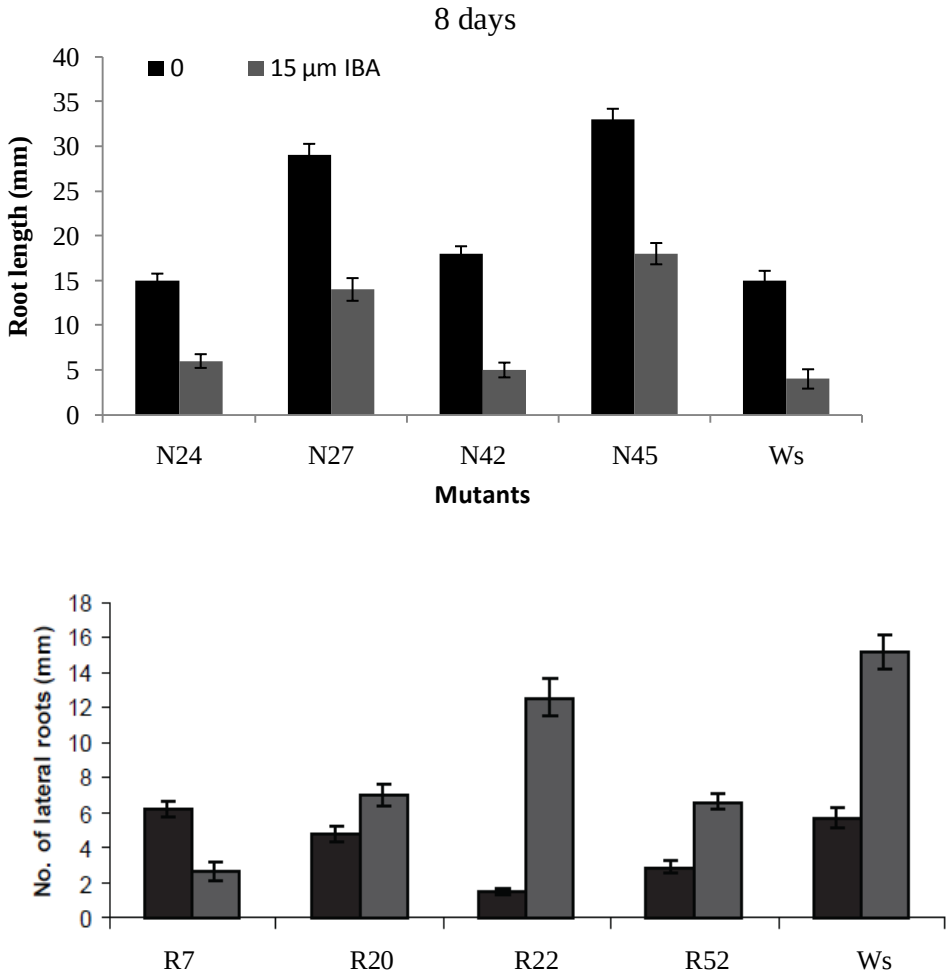


Figure 6-1. R and N mutants response to IBA and sucrose

Root length of 10 d old seedlings grown under continuous light on 0.5% sucrose medium supplemented with the indicated concentration of IBA . Error bars represent the standard error of the means ($n \geq 12$).

Table 6-1. Summary of the R mutants phenotypes

Mutants	IBA resistant	IAA sensitive	Sucrose Dependent
R55	R		
R53	R		
R11	R		
R4	R	S	SD
R7	R	S	SD
R57	R	S	
R62	R	S	
R20	R	S	
R50	R		
R22	R	MAYBE	
R24	R	MAYBE	
R52	R	S	
R82	R	S	
R13	R		
R16	R	S	
R20	R		

6.2. Discussion

It is important to continue screening for peroxisome defective mutants in plants for several reasons. It would be of value to isolate additional peroxisome defective mutants in Arabidopsis because the screen for peroxisome mutants in plants is by no means saturated. To date, 30 PEX biogenesis proteins have been identified in yeast (Saraya et al., 2010, while only 18 PEX biogenesis proteins have been identified and characterized in plants {Nito, 2007 #2490). However, at least 22 *PEX* biogenesis genes were proposed in Arabidopsis genome (Nito et al., 2007). Thus, the mechanism of peroxisome import and recycling in plants has not been completely elucidated. Further, the enzymes involved in various peroxisomal processes in plants such as IBA β-oxidation are still unknown. Therefore, it is important to continue screening for peroxisome mutants in order to elucidate these processes. Additionally, it would be informative to isolate different alleles of the peroxisome mutants we already have. Further, it would be of interest to isolate peroxisome mutants in different Arabidopsis accessions since there is some indication that peroxisomal proteins display accession specific phenotypes.

In this chapter, I conducted a screen on inhibitory concentrations of IBA to isolate additional peroxisome mutants. In the preliminary screen, I isolated several IBA resistant mutants. These mutants were backcrossed to remove unlinked mutations and outcrossed to generate a mapping population. The mapping populations are currently being mapped by Shelly Boyer to identify the mutant genes.

CHAPTER 7: Discussion

Peroxisomes house a number of critical processes in plants (Kaur, 2009). They are the primary site for fatty acid β-oxidation and inactivation of H_2O_2. Peroxisomes play an essential role during germination and early seedling establishment in oilseed plants. Prior to photosynthetic initiation, stored lipids are β-oxidized in peroxisomes to provide the necessary energy for development. Peroxisomes also play a major role in plant hormone β-oxidation. They are the site for the conversion of an auxin storage form, indole-3-butyric acid (IBA), to its active signaling counterpart, indole-3-acetic acid (IAA) (Zolman et al., 2000), JA biosynthesis and fatty acid β-oxidation. The mechanism of peroxisomal matrix protein import and recycling have not been completely elucidated and all the enzymes that act in various peroxisomal processes are not known. Thus, this study provides additional insights into matrix protein import and the roles of the ACX family of enzymes in peroxisomal β-oxidation processes.

Six *ACX* genes have been identified in *Arabidopsis*. *ACX1-ACX4* are all expressed at high levels in Arabidopsis, with *ACX5* showing low, but constitutive expression (Fig. 3-1). The other genes show some expression specificity, especially in floral organs and in response to JA treatment (Fig. 3-1), but each is expressed throughout plant development. The substrate specificity for four ACX proteins has been biochemically characterized *in vitro*. ACX1 catalyzes oxidation of fatty acids with carbon chains of C6:0-C20:0, with highest activity on C12:0-C16:0 (Hooks et al., 1999). ACX2 prefers fatty acids C14:0-C20:0 (Hooks et al., 1999). ACX3 is active on fatty acids C8:0-C14:0 (Eastmond et al.,

2000; Froman et al., 2000), while ACX4 catalyzes the oxidation of short-chain fatty acids C4:0-C6:0 (Hayashi et al., 1999). ACX5 has not been characterized biochemically, but *ACX5* is a duplication of *ACX1*. *ACX6* transcripts have not been detected experimentally and no phenotypes were observed in plants disrupted in *ACX6* (Adham et al., 2005), suggesting that *ACX6* does not play an active role in Arabidopsis fatty acid β-oxidation. Genetic analysis confirms that the biochemical overlap and the similar expression patterns lead to some degree of functional redundancy *in planta*. *acx2*, *acx5*, and *acx6* mutants displayed normal acyl-CoA oxidase activity on all substrates (Adham et al., 2005). Metabolism of long-chain substrates C18:1 and C20:4 was lost in extracts from *acx1* plants (Adham et al., 2005; Pinfield-Wells et al., 2005). *acx3* showed reduced activity on C12:0 acyl-CoA (Eastmond et al., 2000; Adham et al., 2005) whereas oxidation of short-chain substrates (C4:0 and C6:0) was reduced in *acx4* mutant extracts (Rylott et al., 2003; Adham et al., 2005). Despite these alterations in activity, none of the *acx* single mutants has a defect in germination or establishment of seedlings in the transition to photosynthetic adults (Eastmond et al., 2000; Rylott et al., 2003; Adham et al., 2005; Pinfield-Wells et al., 2005). Therefore, to examine the degree of overlap in the ACX enzymes and determine their roles in other peroxisomal β-oxidation processes I generated higher-order mutants, defective in two or three of the *ACX* genes, in the Col-0 accession. I found that an *acx3acx4* double mutant in the Col-0 background is viable with minimal alterations in plant growth and development, although ACX enzyme assays showed only residual short-chain fatty acid β-oxidation. In addition, I generated an *acx1acx3acx4* triple mutant in the Col-0 background and found defects in β-oxidation of long, medium and short-chain fatty acid substrates. The seeds of the triple mutant are

viable and the mutant has only minor disruptions in plant growth and development. The viability of this triple mutant is surprising since ACX1, ACX3 and ACX4 are thought to be the three most active ACX enzymes in fatty acid β-oxidation. Additionally, the other *acx* double and triple mutants in this study show that ACX2 and ACX5 play a role in fatty acid and IBA β-oxidation (Fig. 4-2, 4-3, 4-4). Thus, ACX2 and ACX5 maybe compensating for loss of the other three ACX enzymes in Col-0, since ACX2 is up-regulated in the *acx1acx3acx4* triple mutant. However, this does not answer the question of why the *acx3acx4* in Ws is lethal. Maybe ACX3 and ACX4 in the Ws accession are making a greater contribution to β-oxidation processes, thus, loss of these enzymes causes accumulation of toxic intermediates, resulting in lethality. Some evidence for this can be seen in the *acx3-2acx4-1* mixed double mutant. This double mutant is significantly defective in germination and is dependent on sucrose for seedling establishment (Fig. 3-10; Table 3-1).

Apart from their role in fatty acid β-oxidation, all the *acx* triple mutants showed enhanced IBA insensitivity compared to wild-type, suggesting a role for ACXs in IBA β-oxidation. We are currently attempting to synthesize IBA-CoA to test *acx* mutant extracts. However, the current hypothesis is that the ACXs play a indirect role in IBA β-oxidation. The indirect model for this process suggests that when ACX enzyme activity is reduced, fatty acyl-CoAs are not β-oxidized, thus the CoA pool present in the peroxisome is sequestered by fatty acids thereby decreasing the CoA available to activate IBA to its active form IBA-CoA (Fig. 7-1).

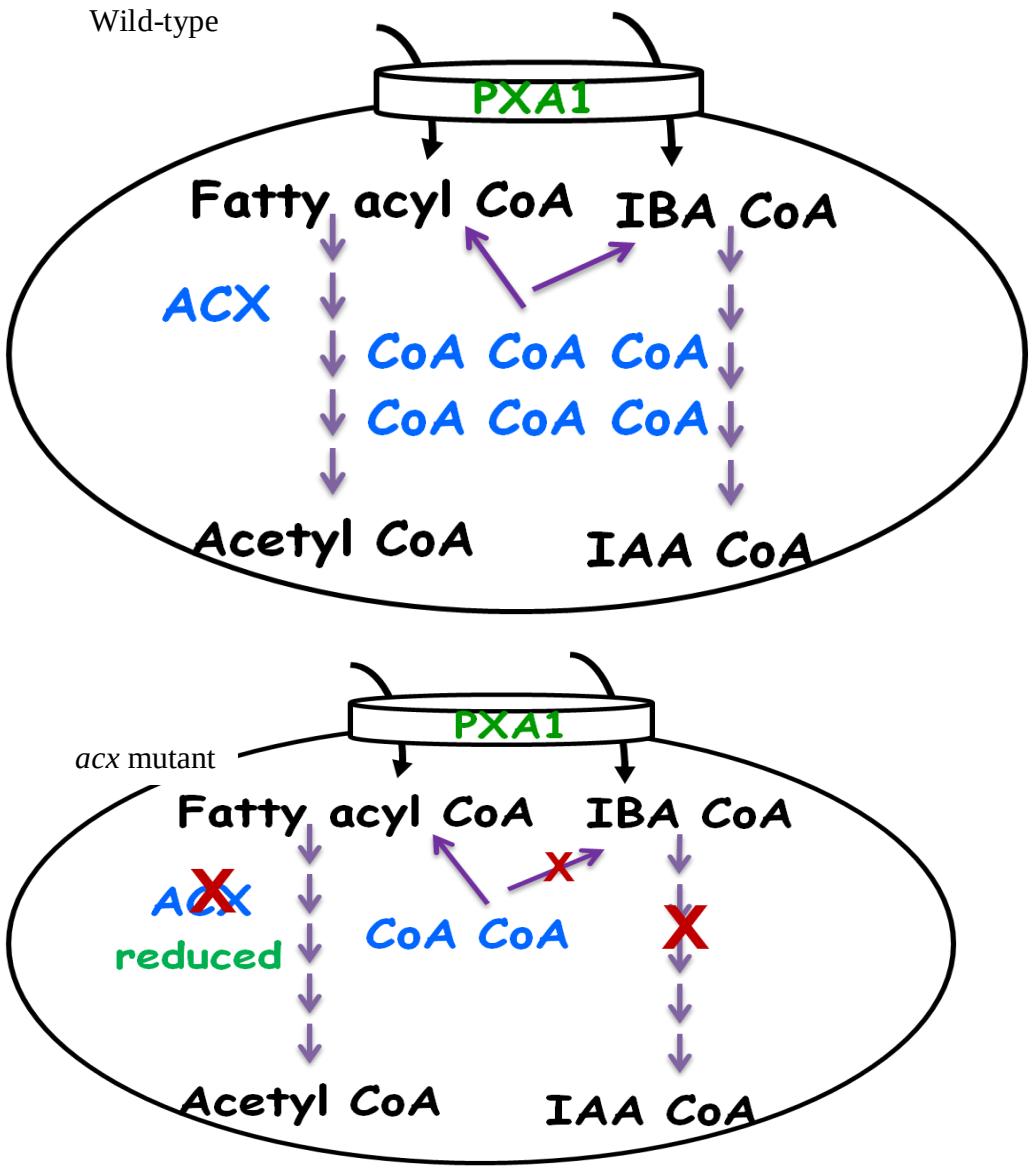


Figure 7-1. Indirect model for how *acx* may block IBA β-oxidation

The processes that occur in peroxisomes depend on import of proteins. PEX5 is a critical player in peroxisomal function. Most peroxisomal matrix proteins contain a PTS1 signal and are directly recognized by the PEX5 receptor (Nito et al., 2002; Reumann, 2004; Reumann et al., 2007). In addition, PTS2 proteins (directly recognized by PEX7) require PEX5 for import (Hayashi et al., 2005); the PEX7 interaction domain has been mapped to the middle region of the Arabidopsis PEX5 protein. Therefore, all peroxisomal matrix-bound proteins require PEX5 activity for import. Previously, the *pex5-1* mutant was identified as an IBA-resistant mutant with weak sucrose dependence and a slight accumulation of seed storage fatty acids (Zolman et al., 2000). *pex5-1* has a C-to-T point mutation, causing a Ser-to-Leu amino acid conversion in region of PEX5 responsible for binding PEX7 (Zolman et al., 2000). *pex5-1* has a PTS2-protein import defect, indicated by reduced GFP-PTS2 peroxisomal localization, but has normal PTS1 import (Woodward and Bartel, 2005). In this work, I characterized phenotypic abnormalities in the *pex5-10* insertion mutant, which has defects in both PTS1 and PTS2 import. Although *pex5-10* exhibits a more severe phenotype than *pex5-1*, I show that the insertion mutant is not a null allele and likely survives because of an unusual T-DNA processing that allows production of a partial transcript. Finally, I created a PTS1-specific *pex5* mutant by complementing *pex5-10* with a partial *PEX5* cDNA, restoring PTS2 import while restricting PTS1 import. Comparison of the *pex5* mutant with mutant transgenic lines expressing the truncated $PEX5_{454}$ proteins allowed me to separate the effects of defects in PTS1, PTS2, or PTS1 and PTS2 import. These mutants provided some insights into the specific pathways affected by loss of either PTS1 or PTS2 proteins.

Genetic, biochemical and phenotypic analysis of peroxisome mutants will continue to provide insights about the importance of these organelles in plant growth and development.

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