Identification and Characterization of Novel Genes and Genetic Interactions that Influence IBA Metabolism

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Identification and Characterization of Novel Genes and Genetic Interactions that Influence IBA Metabolism

by

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B.S., Biology, Missouri State University, 2009

A Dissertation Submitted to The Graduate School at the University of Missouri-St. Louis in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biology with an emphasis in Cell and Molecular Biology

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Abstract

Indole-3-butyric acid (IBA) is an endogenous storage auxin important for maintaining appropriate indole-3-acetic acid (IAA) levels, thereby influencing primary root elongation and lateral root development. IBA is metabolized into free IAA in the peroxisome in a multistep process similar to fatty acid β-oxidation. Although many components specific to IBA metabolism and peroxisome function have been identified, our understanding is incomplete. I sought to identify novel components of IBA metabolism or peroxisome function by conducting a forward genetic screen for *Arabidopsis thaliana* plants with enhanced resistance to IBA.

I identified LONG CHAIN ACYL-COA SYNTHETASE 4 (LACS4) in a screen for enhanced IBA resistance in primary root elongation. LACSs activate substrates by catalyzing the addition of CoA, the necessary first step for fatty acids to participate in β-oxidation or other metabolic pathways. *lacs4* is resistant to the effects of IBA in primary root elongation and dark grown hypocotyl elongation and has reduced lateral root density and these phenotypes are enhanced in *lacs4 ibr3*. The peroxisome localized LACS6 and LACS7 were evaluated for IBA responses and it was discovered that *lacs6*, but not *lacs7*, is resistant to IBA. Both *lacs4* and *lacs6* remain sensitive to IAA in primary root elongation demonstrating auxin responses are intact. Although LACS4 does not possess a canonical peroxisome targeting signal (PTS), it may associate with the peroxisome or be localized to the peroxisome matrix. LACS4 has *in vitro* enzymatic activity on IBA, but not IAA, IAA conjugates, 2-4-DB and disruption of LACS4 activity reduces the amount of IBA-derived IAA *in planta*. I conclude that, in addition to activity on fatty acids, LACS4 and LACS6 also catalyze the addition of CoA onto IBA, the first step in IBA metabolism and a necessary step in generating IBA-derived IAA.
An additional screen for plants with enhanced resistance to IBA in hypocotyl elongation identified six mutants in the ibr3-1 background and five in ibr1-1 background that had tractable phenotypes and were selected for identification and further characterization. Some of the new mutations were successfully separated from the ibr3-1 or ibr1-1 parent backgrounds indicating that defects in these genes result in altered IBA responses independently of ibr3 or ibr1. A combined approach of mapping and whole genome sequencing was used to identify the causative mutation. HRI#55 ibr3-1 and HRI#70 ibr3-1 contained the same causative mutation, a premature stop codon in ACX3. The genomes of HRI#11 ibr1-1 and HRI#41 ibr1-1 were sequenced, but the causative mutations in these enhancers has not yet been identified.

acx3 was identified as the causative mutation in two independent enhancer mutants. acx3 ibr3 has enhanced resistance to IBA in primary root elongation and hypocotyl elongation but is not sucrose dependent suggesting that acx3 further disrupts IBA metabolism but that ibr3 does not disrupt fatty acid β-oxidation. acx3 enhances resistance to IBA when combined with other ibrs while ibr3 acx double mutants vary in strength of IBA responses, suggesting that the ACX proteins do not equally contribute to IBA metabolism. ACX3 does not complement ibr3 when expressed from the IBR3 promoter suggesting that ACX3 does not possess the same enzymatic activity of IBR3 in planta. To investigate the alternative hypothesis that acx3 indirectly disrupts IBA metabolism due to limited CoA availability, ACH2, PXN, and PXA1 were overexpressed in acx3. Overexpression of these genes did not rescue the phenotype of acx3. Although not conclusive, together this data suggest that ACX3 may influence IBA metabolism indirectly.
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Chapter 1: Introduction

1.1. Peroxisomes

Peroxisomes are dynamic multifunctional organelles found in all eukaryotes with diverse functions. They are named after their ability to inactivate hydrogen peroxide because they contain catalase. Their function, size, shape, and number can vary between organisms and cell type and can respond to cues. Although peroxisomes are small, they may contain upwards of 300 proteins (Reumann et al. 2009) and host metabolic processes with diverse functions including, but not limited to, fatty acid β-oxidation, reactive oxygen species (ROS) metabolism, synthesis of secondary metabolites, hormone synthesis, amino acid and purine degradation, stress responses, and defense against pathogens (Smith and Aitchison 2013; Islinger et al. 2018; Kao et al. 2018). Peroxisomes have previously been termed microbody, glyoxysome, and gerontosome to distinguish their specialized roles in particular tissues or developmental stages (Pracharoenwattana and Smith 2008).

Plant peroxisomes are unique from those of animals in that they are the sole location of fatty acid β-oxidation, synthesize three phytohormones, and participate in photorespiration (Hu et al. 2012; Smith and Aitchison 2013). Plant peroxisomes are the sole location of fatty acid β-oxidation, making this organelle indispensable to early plant development as seedlings must rely on the breakdown of lipids to generate all the energy for growth before they become capable of utilizing sunlight for photosynthesis (Graham 2008). Peroxisomes house enzymes necessary for a diverse array of metabolic processes making them vital throughout a plant’s life.
1.1.a. Peroxisome biogenesis

Peroxisomes are distinct entities with functions that separate them from other organelles. The mechanism of peroxisome biogenesis has been debated for decades. It is widely accepted that peroxin (PEX) proteins are required for proper peroxisome assembly, maintenance, and function across species (Hu et al. 2012; Agrawal and Subramani 2016; Kao et al. 2018; Farré et al. 2019; Su et al. 2019), but the endoplasmic reticulum (ER) has continually been implicated in peroxisome biogenesis although the exact mechanism has yet to be elucidated.

Peroxisomes have a close relationship with the ER. The two organelles work together to synthesize specific lipids in humans (Schuldiner and Zalckvar 2017), are often physically located in close proximity (Nivikoff and Shin 1964; Huang et al. 1983; Barton et al. 2013), and have recently been demonstrated to be tethered together (Schuldiner and Zalckvar 2017). There is evidence that peroxisomes are derived from the ER and is supported by the close relationship of the ER and peroxisomes. There are several models describing possible mechanisms of peroxisome biogenesis: the autonomous model, the ER semi-autonomous model, and the ER vesiculation model (Hu et al. 2012).

In the autonomous model, peroxisomes are derived from pre-existing peroxisomes through a process of growth and division similar to mitochondria and chloroplasts (Lazarow and Fujiki 1985; Hu et al. 2012; Agrawal and Subramani 2016). Peroxisomes divide into new peroxisomes by fission with the actions of PEX11, FISSION1, and dynamin-related proteins (DRP) (Hu et al. 2012; Smith and Aitchison 2013; Kao et al. 2018; Farré et al. 2019). This model is supported by the observation that peroxisome matrix proteins are synthesized in the cytosol and are imported into the peroxisome
This demonstrates that new and functional peroxisomes can be generated without the assistance of the ER. However, it is hypothesized that the ER serves as the source of membrane lipids to help grow peroxisomes as the ER is the location of most lipid synthesis and peroxisomes are often seen nearby.

In the ER-vesiculation model, peroxisomes are created de novo by budding off the ER membrane to become independent entities. Analysis of several mutant yeast species that lacked peroxisomes revealed that upon complementation of the mutant gene, peroxisomes were regenerated (Subramani 1998), demonstrating that new peroxisomes can be made without pre-existing peroxisomes. A similar observation was seen in a human cell line derived from a patient with Zellweger syndrome, caused by peroxisome dysfunction, which was devoid of obvious peroxisomes. Peroxisome synthesis could be restored by expressing PEX16 (South and Gould 1999). These observations strongly support that peroxisome biogenesis can occur de novo and does not require pre-existing peroxisomes. Other evidence for this model is that some peroxisome membrane proteins (PMP) are trafficked to the peroxisome by way of the ER. This is seen across systems including yeast, mammals, and plants (Hu et al. 2012; Agrawal and Subramani 2016). In plants, the peroxisome membrane proteins ascorbate peroxidase (APX), PEX10, and PEX16 have been detected in both the ER and peroxisomes (Lisenbee et al. 2003; Hu et al. 2012; Agrawal and Subramani 2016). Although there is no direct evidence for de novo peroxisome synthesis off the ER in plants, evidence from mammals and yeast and localization of proteins destined for the peroxisome in the ER suggest it is possible.

The current working model in plants is the semi-autonomous model. In this model, peroxisomes can arise de novo off the ER, then grow and divide to generate more
peroxisomes (Mullen and Trelease 2006; Hu et al. 2012; Su et al. 2019). Peroxisomes may bud off of specialized peroxisome ER (pER) domains of the ER where PMPs destined for the peroxisome accumulate (Mullen and Trelease 2006; Hu et al. 2012). The pER was suggested as a mechanism because APX was detected in distinct regions of the ER instead of throughout the organelle and appears to contain a targeting signal for this specialized ER localization (Mullen and Trelease 2000; Mullen and Trelease 2006).

Although conclusive evidence for one model of peroxisome biogenesis in plants is not available, much evidence exists that the ER has a close relationship with peroxisomes and is involved in peroxisome biogenesis.

1.1.b. Peroxisome import

Peroxisomes do not contain DNA, therefore, all proteins and components destined for the peroxisome are actively imported from the cytosol. Proteins destined for the peroxisome matrix typically contain one of two peroxisome targeting signals (PTS): PTS1, a C-terminal tripeptide with the consensus amino acids [S][RK][LM], or PTS2, a R[LI]X₅HL near the N-terminus (Reumann 2004; Lanyon-Hogg et al. 2010). Proteins containing a PTS1 or PTS2 are recognized by their receptors PEX5 or PEX7, respectively, in the cytosol. PEX5 and PEX7 allow the cargo protein to be docked with the peroxisome membrane via interactions with other PEX proteins and released into the peroxisome matrix (Hu et al. 2012; Kao et al. 2018).

Most proteins destined for the peroxisome matrix are imported by the canonical PTS and receptor mediated import, however, several proteins are imported through other mechanisms. Peroxisomes are able to import folded proteins and even oligomers (Walton et al. 1995; Brown and Baker 2008). All subunits of an oligomer destined for the
peroxisome can be imported even when not every subunit contains a PTS (Lee et al. 1997; Brown and Baker 2008), illustrating that proteins being bound to a PTS containing protein is sufficient for peroxisome import. Subunits of protein phosphatase 2A are imported into the peroxisome via this piggybacking mechanism in Arabidopsis (Kataya et al. 2015). Some proteins exploit this by piggybacking onto PTS containing proteins even if they are not part of an oligomer (Thoms 2015). This piggybacking mechanism has also been reported for proteins that do not function as a component of a complex, including Nicotinamidase 1 which is co-imported into the peroxisome with glycerol-3-phosphate dehydrogenase 1 in Saccharomyces cerevisiae (Saryi et al. 2017).

Substrates and small molecules, such as cofactors and metabolites necessary for the metabolic processes housed within the peroxisome, are actively imported through transport proteins (Linka and Esser 2012). β-oxidation substrates are imported by PEROXISOMAL ABC TRANSPORTER PXA1/PED3/CTS (PXA1) (Zolman et al. 2001; Hayashi et al. 2002; Linka and Esser 2012), making PXA1 crucial for proper oil utilization and hormone production. ATP is imported from the cytoplasm and into the peroxisome via the peroxisomal adenine nucleotide carriers (PNC), PNC1 and PNC2 (Arai et al. 2008; Linka et al. 2008) while NAD⁺ is transported through the peroxisomal NAD⁺ transporter, PXN (Agrimi et al. 2012; Bernhardt et al. 2012; van Roermund et al. 2016). A model of peroxisome import is depicted in Figure 1-1.

1.1.c. Peroxisome function

Peroxisomes participate in a diverse array of metabolic and catabolic processes. In plants, the organelles participates in photorespiration, fatty acid β-oxidation, detoxifies reactive oxygen species (ROS), participates in phytohormone synthesis, and are sources
of signaling molecules. Housing these pathways make peroxisomes important for germination, seedling establishment, development, stomatal opening, fertility, defense, senescence, and responses to environmental cues and biotic and abiotic stresses (Hu et al. 2012; Reumann and Bartel 2016; Kao et al. 2018; Su et al. 2019).

Peroxisomes are essential for generating energy for plants under developmental conditions in which photosynthesis is absent or limited such as germination and senescence. In oilseed plants, fatty acids are metabolized into acetyl-CoA which can enter the TCA cycle or the glyoxylate cycle to ultimately generate sugars that support plant growth (Eastmond and Graham 2001; Graham 2008). Enzymes necessary for fatty acid β-oxidation and the glyoxylate cycle are contained within the peroxisome (Eastmond and Graham 2001; Hu et al. 2012; Kao et al. 2018). Peroxisomes have been called glyoxysomes to capture their specialized role in oilseed plants (Del Río et al. 1998; Eastmond and Graham 2001). Plant peroxisomes are the sole location of fatty acid β-oxidation making them essential for germination during early seedling development.

Peroxisomes were named for their ability to inactivate hydrogen peroxide. Peroxisomes contain catalase, APXs, and peroxiredoxins to neutralize hydrogen peroxide and other ROS (Kao et al. 2018; Su et al. 2019). Because of this unique ability to neutralize damaging ROS, metabolic pathways that generate ROS byproducts are contained within the peroxisome. Photorespiration and fatty acid β-oxidation, pathways essential throughout the life of a plant, are two metabolic pathways that generate significant H₂O₂ (Kao et al. 2018; Su et al. 2019; Corpas et al. 2020), and are therefore restricted to this organelle. Peroxisomes may also scavenge for ROS.
conditions resulting in increased accumulation of ROS can trigger peroxisome proliferation to mitigate cellular damage (Su et al. 2019).

Peroxisomes participate in metabolism of other reactive species such as reactive nitrogen species (RNS) and reactive sulfur species (RSS) (Corpas et al. 2020). Evidence suggests RNS and RSS function as signaling molecules as they accumulate after stresses (Kao et al. 2018; Corpas et al. 2020). Nitric oxide (NO), one of the most studied RNS, influences lateral root formation, pollen germination, senescence, and responses to environmental stresses (Corpas et al. 2020).

In leaves, peroxisomes have a prominent role in photorespiration. Photorespiration is a salvage pathway to rectify incidences when ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisco) consumes O$_2$ and generates CO$_2$ instead of consuming CO$_2$ (Hu et al. 2012; Kao et al. 2018). Enzymes that participate in photorespiration span chloroplasts, peroxisomes, and mitochondria. Peroxisomes contain the enzymes glycolate oxidase, two aminotransferases, and malate dehydrogenase that directly participate in catalyzing photorespiration reactions (Reumann and Weber 2006; Hu et al. 2012). Photorespiration is essential for plants and mutations in core photorespiration enzymes are often lethal (Hu et al. 2012). It is estimated that photorespiration generates approximately 70 % of total H$_2$O$_2$ production (Corpas et al. 2020), explaining why catalase is the most abundant protein found in leaf peroxisomes (Reumann and Weber 2006). Peroxisomes are essential in photosynthetic tissue to directly catalyze reactions in photorespiration and to neutralize the substantial amount of toxic byproducts produced.
Peroxisomes also participate in synthesis of three hormones: indole-3-acetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA). A storage auxin, indole-3-butyric acid (IBA), undergoes one round of β-oxidation in the peroxisome to generate IAA. IBA-generated IAA influences many aspects of root architecture (Frick and Strader 2018). JA is synthesized by the β-oxidation of 12-oxo-phytodienoic acid (OPDA) and is important for fertility and responses to stress (Wasternack and Hause 2013). Peroxisomes contribute to SA synthesis by β-oxidizing cinnamic acid to benzoic acid which is converted into SA in the cytosol (Kao et al. 2018).

1.1.d. Peroxisome β-oxidation pathways

Peroxisomes house enzymes for three crucial β-oxidation pathways: fatty acid β-oxidation, JA synthesis, and IBA conversion to IAA (Hu et al. 2012). These β-oxidation pathways and depicted in Figure 1-1. In plants, the peroxisome is the sole location of fatty acid β-oxidation. Long-chain fatty acids are imported into peroxisomes through PXA1 (Zolman et al. 2001; Hayashi et al. 2002). Fatty acids are broken down two carbons per cycle to produce acetyl-CoA, which can enter the citric acid cycle to ultimately be oxidized for energy production (Hu et al. 2012). Peroxisomes are, therefore, crucial for early development and seedling establishment because plants rely on the energy produced by the breakdown of fatty acids stored as triacylglycerol (TAG) before they are photosynthetically active (Graham 2008).

Following substrate import, fatty acid β-oxidation begins with the addition of CoA onto fatty acids by LONG CHAIN ACYL-COA SYNTHETASE 6 (LACS6) and LACS7 (Fulda et al. 2004). CoA addition is necessary to activate the fatty acid for recognition before metabolism proceeds. Fatty acyl-CoA esters are oxidized by acyl-CoA
oxidases (ACX), hydrated, and then dehydrogenated by the multifunctional proteins MFP2, and AIM1, and cleaved by the thiolase, PED1/KAT2/KAT5, to release acetyl-CoA and a shortened fatty acid (Graham 2008; Hu et al. 2012). This process can be repeated as needed. For instance, very long-chain fatty acids, as found in seed storage lipids, enter the pathway multiple times to release many molecules of acetyl-CoA.

In a similar process, JA is the product of three rounds of β-oxidation of OPDA, making peroxisomal activity important for JA effects including fertility and response to abiotic and biotic stressors (Wasternack and Hause 2013). OPDA is imported into the peroxisome through PXA1, reduced to OPC8:0 by OPR3, activated with CoA by OPCL1 (Koo et al. 2006; Kienow et al. 2008) and β-oxidized by actions of ACX1, ACX5, AIM1, and PED1/KAT2 (Hu et al. 2012; Wasternack and Hause 2013; Wasternack and Strnad 2018).

The third β-oxidation pathway housed in the peroxisome is the conversion of IBA into IAA, hypothesized to be mechanistically related to fatty acid β-oxidation. IBA is imported through PXA1 and metabolized through the actions of IBR3 and/or ACXs, predicted oxidases, IBR10, a predicted hydratase, and IBR1, a predicted dehydrogenase to release IAA (Zolman et al. 2007; Zolman et al. 2008; Hu et al. 2012). Peroxisomes contribute to the pool of free IAA by β-oxidizing IBA making this organelle important for auxin homeostasis (Spiess and Zolman 2013).

Peroxisomes contain fatty acid, IBA, and JA β-oxidation pathways, but it is unclear if peroxisomes globally contain all three pathways at all times, in all tissues, or all cell types. It is possible that the matrix of individual peroxisomes varies allowing for peroxisomes variants to be more specialized for a particular β-oxidation pathway at
specific developmental times, specific tissues, or in specific cells. However, the peroxisome matrix may be uniform throughout the plant and utilization of the contained pathways is dependent upon delivery of substrates which may be tissue specific.
Peroxisomes must import all proteins, substrates, and cofactors. Substrates are imported through PXA1, ATP is imported through PNC1 and PNC2, while NAD$^+$ is transported through PXN. Proteins destined for the peroxisome matrix contain a PTS1 or PTS2. PTS containing proteins dock with the appropriate carrier protein, PEX5 or PEX7, and are imported with the aid of additional PEX proteins. PTS cargo is unloaded into the peroxisome matrix and PEX5 and PEX7 are recycled to the cytoplasm.

Peroxisomes house three crucial β-oxidation pathways: IBA conversion to IAA, fatty acid β-oxidation, and JA synthesis. Enzymatic steps in these β-oxidation pathways are the same but may be carried out by enzymes unique to that pathway or common between several or all of the pathways. It is unclear if peroxisome content is uniform or specialized throughout plant tissues and cells.
1.2. Auxin and IBA

Auxin is a hormone that has been implicated in almost all plant growth and developmental processes. Auxin signaling controls plant cell elongation, division, tropisms, tissue patterning, and cell differentiation and is, therefore, crucial for development throughout the life span of a plant. In addition, auxin is controlled by or controls other hormones which increases the diversity of auxin’s effects (Woodward and Bartel 2005a).

1.2.a. Regulation of IAA levels

Due to its diverse and extensive effects, proper levels of IAA, the primary signaling auxin, are tightly controlled through synthesis, degradation, transport, and sequestration (Korasick et al. 2013; Spiess et al. 2014). Auxin is believed to be primarily synthesized \textit{de novo} from tryptophan but there is evidence that IAA can also be synthesized via a tryptophan-independent pathway (Woodward and Bartel 2005a; Korasick et al. 2013). Auxin can be sequestered in inactive forms by conjugation to amino acids or side chain elongation to form IBA. IAA conjugated to a number of amino acids and sugars exist in plants (Korasick et al. 2013). In addition, IAA exists in a methyl-ester form, me-IAA (Korasick et al. 2013). Free IAA can more readily be made from IAA storage forms than by \textit{de novo} synthesis as IAA conjugates are converted back to IAA with a single hydrolysis reaction (Korasick et al. 2013). IBA is structurally similar to IAA but the side chain is two carbons longer; IBA is metabolized to IAA in the peroxisome in a multistep process (Hu et al. 2012). These auxin input pathways ensure a plant has an appropriate amount of auxin and can readily increase IAA levels when
necessary. Plants with combined mutations in IAA-conjugate hydrolysis and IBA metabolism have phenotypes associated with reduced auxin levels and increase expression of auxin biosynthesis genes to mitigate the low auxin levels (Spiess et al. 2014). These unique input pathways vary between individual cells, organs, and developmental stages, but all function to ensure proper levels of this crucial hormone.

It is also important that plants do not have auxin in excess. When IAA levels are elevated, IAA can be reversibly or irreversibly conjugated to other molecules, converted to IBA, or oxidized (Woodward and Bartel 2005a; Korasick et al. 2013). Seedlings treated with exogenous auxin can conjugate IAA to aspartic acid and glutamic acid. These conjugates, IAA-Asp and IAA-Glu, are not well hydrolyzed back to IAA and are hypothesized to primarily serve as terminal inactivation pathways (Korasick et al. 2013). IAA is also irreversibly oxidized to ox-IAA and targeted for degradation (Korasick et al. 2013). There is evidence that when IAA levels are high, IAA terminal conjugation pathways are upregulated while IAA-storage forms are downregulated (Woodward and Bartel 2005a). A model of IAA homeostasis is depicted in Figure 1-2.

The numerous forms of IAA stand as evidence that plants tightly regulate auxin levels. This delicate balance of auxin homeostasis is crucial for plants to both have just enough auxin to grow and quickly respond to stimuli without maintaining excess.

1.2.b. Role of IBA

IBA-derived auxin influences cotyledon expansion, apical hook formation, and several aspects of root architecture, including primary root elongation, lateral root development, root hair elongation, root apical meristem size, adventitious root development, and root hair elongation in response to phosphate starvation (Korasick et al.
IBA has dramatic effects on root architecture when applied exogenously, stimulating plants to develop a more branched root system. IBA is more stable than IAA, more effective at stimulating lateral root initiation, and has become widely used in horticulture for those reasons. IBA is the active ingredient in commercial rooting agent. Recent evidence suggests that IBA to IAA metabolism in the root tip is the primary source of auxin to initiate lateral root development (De Rybel et al. 2012; Xuan et al. 2015) and reinforces the auxin gradient to elongate lateral roots (Strader and Bartel 2011). These developmental roles are hypothesized to be due to the actions of IBA-derived IAA instead of direct effects of IBA.

IBA is hypothesized to be an inactive storage form of auxin. The side chain of IBA is two carbons longer than IAA. This elongated side chain is thought to be the reason that IBA cannot bind to the TIR1–Aux/IAA co-receptor active site (Uzunova et al. 2016), thereby preventing IBA from stimulating auxin signaling on its own. IAA binding to the TIR1–Aux/IAA co-receptor initiates transcriptional responses that are responsible for IAA effects. Genetic analysis of mutants resistant to IBA support that IBA must be converted into IAA for activity.

IBA is an important source of free IAA. Combination mutants disrupting IBA to IAA conversion and IAA-conjugate hydrolases had phenotypes consistent with reduced auxin levels including increased primary root length, decreased lateral root density, decreased root hair length, vein patterning defects, abnormal leaf morphology, and novel germination delays (Spiess et al. 2014). These mutants had reduced free IAA levels when compared with the single mutant parents and demonstrated that IAA storage forms are
physiologically relevant sources of auxin in planta. This data demonstrates that IBA is an important storage form of auxin that contributes to the free IAA pool which influences plant development from seedling through adult stages.

1.2.c. IBA storage and transport

Although IBA is thought to be inactive, it can be further regulated by transport and conjugation (Figure 1-2). Long distance movement of radiolabeled IBA has been detected in Arabidopsis (Ludwig-Müller et al. 1995) and it appears to be transported by designated transporters distinct from those for IAA. IBA transport is not altered in auxin transporter 1 (aux1) and there is no evidence that IBA is effluxed via the IAA transporters PIN2, PIN7, ABCB1, or ABCB19 (Strader and Bartel 2011; Damodaran and Strader 2019). Three IBA transporters have identified, aside from PXA1. IBA efflux carriers, ABCG37 and ABCG36, were identified in a screen for plants with increased sensitivity to IBA and were discovered to transport IBA, but not IAA, out of the root (Strader and Bartel 2011; Damodaran and Strader 2019). TRANSPORTER OF IBA 1 (TOB1) was identified as an IBA transporter that localizes to the vacuole membrane (Michniewicz et al. 2019). TOB1 may function to sequester IBA into the vacuole in efforts to limit lateral root development and may be regulated by another hormone, cytokinin (Michniewicz et al. 2019). ABCG37, ABCG36, and TOB1 transport IBA out of the plant or into a compartment making IBA unusable to the plant. The functions of these transporters appear to limit the amount of IBA available to the plant which suggests the levels of IBA are regulated. Transport of IBA is not the only mechanism to limit the amount of available IBA. IBA can be conjugated to glucose and amino acids similarly to IAA.
IBA conjugates have been detected in plants. IBA can be conjugated to glucose by UGT74E2. IBA can also be conjugated to amino acids, glycine, and alanine. An IAR3 auxin amidohydrolase gene (TaIAR3) was identified in wheat that can hydrolyze IBA-Ala and IBA-Gly (Campanella et al. 2004) suggesting that these forms of IBA also serve as auxin storage forms. A GRETCHEN HAGEN 3 (GH3) acyl acid amido synthetase, GH3.15, has high specificity for IBA and conjugates IBA to aspartate. When overexpressed, GH3.15 displays classic phenotypes of IBA resistance in primary root elongation and lateral root density (Sherp et al. 2018), supporting that IBA-conjugates regulate the availability of IBA.

The identification of IBA transporters and enzymes acting on IBA-conjugates suggests that the levels of IBA available is regulated and sophisticated. Fine-tuned regulation of IBA may be necessary for a plant to properly control root development. Transport and conjugation of IBA can help modulate the amount of IBA available to be β-oxidized into IAA to specific tissues and developmental time points, or in response to environmental signals.

1.2.d. Identification of IBA resistant mutants

Forward genetics has been a successful technique to identify genes that are important for IBA metabolism or peroxisome function. Many mutant seedlings were found to be resistant to IBA in primary root elongation in screens (Zolman et al. 2000; Zolman et al. 2007; Zolman et al. 2008). These mutants could be separated into categories based on secondary phenotypes in addition to resistance to IBA. Several mutants had additional phenotypes such as sucrose dependence, and resistance to the synthetic auxins 4-(2,4-Dichlorophenoxy)butyric acid (2-4-DB) and 2,4-
Dichlorophenoxyacetic acid (2-4-D). Mutants that had differential responses on these treatments and were divided into four classes (Zolman et al. 2000). The pleiotropic and differential phenotypes suggest the defective genes have function in additional processes other than IBA metabolism or a different mechanism of action from each other.

Some mutants appeared specific to IBA metabolism based on the secondary phenotypes and were termed IBA-response mutants (ibr). From this mutant class, three enzymes, ibr3, ibr10, and ibr1, were identified that are hypothesized to directly catalyze IBA to IAA conversion (Zolman et al. 2007; Zolman et al. 2008). IBR3, the predicted oxidase, IBR10, the predicted hydratase, and IBR1, the predicted dehydrogenase, are thought to act in sequential steps to catalyze IBA conversion to IAA in a mechanism similar to that of fatty acid β-oxidation (Zolman et al. 2007; Zolman et al. 2008). IBR3, IBR10, and IBR1 appear specific to IBA metabolism as mutants do not have phenotypes associated with defects in fatty acid β-oxidation, JA synthesis, or peroxisomal import (Zolman et al. 2007; Zolman et al. 2008).

Several mutants were sucrose dependent, suggesting that they also disrupted fatty acid β-oxidation or general peroxisome function. From this class, pex4, pex5, pex6, pex7, and pxa1 were identified (Zolman et al. 2000; Zolman et al. 2001a; Zolman and Bartel 2004; Woodward and Bartel 2005b; Zolman et al. 2005). PEX5, PEX7, and PXA1 function in protein import into the peroxisome (Zolman et al. 2001a; Woodward and Bartel 2005b; Khan and Zolman 2010; Nyathi et al. 2010). PEX4, a ubiquitin-conjugating enzyme (Zolman et al. 2005; Lingard et al. 2009), and PEX6, an AAA ATPase (Zolman and Bartel 2004; Gonzalez et al. 2017), have roles in peroxisome protein cycling and import. Mutations to PEX4, PEX5, PEX6, PEX7, and PXA1 affect not only IBA
metabolism, but all peroxisome metabolic processes explaining their pleiotropic phenotypes.

Mutations to known fatty acid β-oxidation enzymes, including *aim1* and *ped1*, have IBA-response phenotypes as well (Hayashi et al. 1998; Zolman et al. 2000); these phenotypes could indicate redundant enzymatic activity, with multiple enzymes acting on IBA at each step, or enzyme activity on multiple substrates affecting both pathways.

Alternatively, IBA-resistant responses in the mutants *acx1*, *acx3*, *chy1*, and *ech2* suggest indirect effects such as CoA limitations (Adham et al. 2005) or accumulation of toxic intermediates (Zolman et al. 2001b; Li et al. 2019) also influence peroxisomal metabolic pathways.

From screens for seedlings resistant to IBA, a wide range of genes important for IBA metabolism and peroxisome function have been identified. These studies provide the foundational evidence that IBA metabolism occurs in the peroxisome and that a functional organelle is required. IBA metabolism requires that the peroxisome must actively import functional proteins, substrates, and cofactors necessary for the contained metabolic processes (Hu et al. 2012). IBA-resistant phenotypes can be caused by mutations specific to IBA metabolism, such as enzymes that directly catalyze steps in IBA β-oxidation (Zolman et al. 2007; Zolman et al. 2008), as well as indirectly, by mutations that compromise internal peroxisome dynamics (Adham et al. 2005), generation of toxic intermediates (Zolman et al. 2001b; Li et al. 2019), or by disrupting function of the organelle as a whole (Zolman et al. 2001a). These studies are the foundation of our current knowledge of IBA metabolism and have also provided critical information about general peroxisome function.
1.2.e. IBA to IAA conversion

Analysis of *ibr* mutants suggests IBA is β-oxidized into IAA in a stepwise process paralleling fatty acid β-oxidation, the process in which two carbons are removed from fatty acids (Hu et al. 2012). IBA is thought to be imported into the peroxisome to undergo one round of β-oxidation to remove the two-carbon elongation, producing IAA. Current genetic evidence suggests IBA β-oxidation occurs with the action of IBR3, a predicted oxidase, IBR10, a predicted hydratase, and IBR1, a predicted dehydrogenase, to release IAA (Zolman et al. 2007; Zolman et al. 2008).

The CoA synthetase that would function as the first step to charge IBA with CoA is currently unknown (Figure 1-1). Therefore, enzymatic activity of IBR3, IBR10, and IBR1 has not been tested due to inability to make IBA-CoA *in vitro*. Attempts to synthesize IBA-CoA *in vitro* or through forced activity of a nonspecific CoA synthetase have been unsuccessful. Until the enzymatic activity of the IBRs can be tested, only genetic evidence exists that they metabolize IBA.

IBA is thought to only be a storage auxin and must be β-oxidized into IAA to elicit effects. IBA has dramatic influences on root architecture and morphology by influencing primary root length, root branching, and root hair length (Frick and Strader 2018). Plants with mutations to IBR3, IBR10, or IBR1 have increased primary root length, reduced lateral root density, and reduced root hair length when stimulated with exogenous IBA (Zolman et al. 2007; Zolman et al. 2008; Frick and Strader 2018). IBA is hypothesized to be the main form of auxin to stimulate lateral root development (De Rybel et al. 2012; Xuan et al. 2015) making IBA β-oxidation crucial for proper development of root systems in plants.
Proper levels of IAA are maintained through a balance of input pathways. IAA is synthesized *de novo* from tryptophan and tryptophan independent pathways. IAA can be reversibly held in inactive storage forms such as IBA and IAA-conjugates. IBA can also be reversibly conjugated to amino acids. IAA is degraded by irreversible conversion to oxIAA.

**Figure 1-2. Pathways to maintain IAA homeostasis.**

Proper levels of IAA are maintained through a balance of input pathways. IAA is synthesized *de novo* from tryptophan and tryptophan independent pathways. IAA can be reversibly held in inactive storage forms such as IBA and IAA-conjugates. IBA can also be reversibly conjugated to amino acids. IAA is degraded by irreversible conversion to oxIAA.
1.2.f. Open questions

Although research in the last two decades has revealed much about the role of IBA in plant development, much is still not known or well understood. It is not known how plants synthesize IBA. Evidence from maize suggests IBA can by synthesized from IAA (Ludwig-Müller and Epstein 1991; Ludwig-Müller and Epstein 1992) and *in vitro* assays suggest this activity is associated with the microsomal fraction (Ludwig-Muller and Hilgenberg 1995); however, the exact enzyme that converts IAA to IBA has not conclusively been documented. IBA synthetase activity has been detected in *Arabidopsis* (Ludwig-Müller 2007), but no mutant has been linked to a defect in IBA synthesis.

Association of IBA synthetase activity with the ER membrane also raised questions about intracellular IBA localization, as there is no current evidence of ER localized pools of IBA or an ER localized IBA transporter. IBA association with the peroxisome is known because of extensive work on the *ibr* mutants. IBA transport into the vacuole is a recent discovery, was unexpected, and suggests there may be intracellular pools of IBA with the potential for discrete functions.

IBA influences numerous plant developmental processes (Frick and Strader 2018) and contributes to the overall auxin pool (Spiess et al. 2014). However, it is unclear the spatial and temporal contributions of IBA to each developmental process and environmental responses. For instance, IBA is thought to be the major source of auxin for plants to initiate lateral roots during development (De Rybel et al. 2012; Xuan et al. 2015) but has not conclusively been linked to increases in root branching that occurs when plants encounter stress conditions. It is unclear if plants utilize IBA only during development or also to modulate growth throughout its life cycle.
All enzymes necessary for IBA β-oxidation have not yet been identified; the CoA synthetase initiating IBA β-oxidation is unknown and has inhibited studying the enzymatic action of the downstream enzymes. Hypothesized actions of IBRs are suggested due to their predicted enzymatic actions and phenotypes, but their activity has never been assayed in vitro. In addition, it is not known how IAA is transported out of the peroxisome as a peroxisomal IAA efflux carrier has not been identified (Frick and Strader 2018; Damodaran and Strader 2019).

1.3. Overview of study

Although many components specific to IBA metabolism and peroxisome function have been identified, our understanding is incomplete. For instance, the CoA synthetase initiating IBA β-oxidation and a peroxisomal IAA efflux carrier have not been identified (Hu et al. 2012; Damodaran and Strader 2019). I sought to identify additional components of IBA metabolism by continuing the forward genetic screen for IBA resistant plants.

The screen for IBA resistance is not yet saturated but often identifies new alleles of known IBA resistance genes instead of genes novel to IBA metabolism. To enhance my chances for a novel discovery, ibr3-1 and ibr1-1 were remutagenized and screened for enhanced resistance to IBA in dark grown hypocotyl elongation. The original forward genetic screen identified mutants with IBA resistance to root elongation. A screen for dark grown hypocotyls instead of roots and inclusion of a mutant background serves to differentiate this screen from the original. This screen could identify genes important for IBA to IAA conversion, important for function of IBR3 or IBR1, or general peroxisome function in Arabidopsis thaliana.
In Chapter 3, I describe the identification of LONG-CHAIN ACYL-COA SYNTHETASE 4 (LACS4) from an enhancer screen with an ibr3 mutant background. lacs4 enhances the IBA resistance of ibr3 and is resistant to IBA on its own. LACS4 and its relationship with IBA was not previously known. The lacs4 mutant was phenotypically characterized for responses to IBA, IAA, and synthetic auxins and for defects in fatty acid β-oxidation. I discovered that LACS4 may localize to the peroxisome even though it does not possess a PTS. I assayed LACS4 for activity on IBA and structurally similar auxins and discovered LACS4 has activity on IBA but not IAA in vitro. The mutant phenotypes and enzymatic activity of LACS4 suggest that it functions as the initial step in IBA metabolism, charging IBA with CoA allowing entry into the β-oxidation process. From studying LACS4, I also determined that LACS6 may work in concert with LACS4 to charge IBA with CoA, as lacs6 is resistant to IBA. This discovery completes the enzymatic components that metabolize IBA to IAA.

Eleven mutants were pulled from the enhancer screen for further characterization. The screen and initial characterization of mutants is described in Chapter 4. I attempted to identify causative mutations by using whole genome sequencing in combination with rough mapping. One mutant was identified as being defective in ACX3. All other mutants remain unidentified.

It has been previously documented that acx3 is resistant to IBA and enhances the resistance of ibr3 (Adham et al. 2005; Zolman et al. 2007), although the role of ACX3 in IBA metabolism remains unclear. In Chapter 5, I examine if ACX3 directly or indirectly influences IBA metabolism using genetic techniques. The hypothesis of ACX3 directly functioning as an oxidase in IBA metabolism was tested by in planta complementation.
ACX3 could not complement *ibr3* even when expressed from the IBR3 promoter, suggesting ACX3 does not directly function in IBA metabolism. The hypothesis of ACX3 disrupting IBA metabolism indirectly was tested by perturbing peroxisome CoA levels by overexpressing peroxisome import proteins. Results from these experiments were inconclusive but demonstrated that peroxisome metabolic pathways might be sensitive to excessive influx of molecules and can be disrupted indirectly.
Chapter 2. Materials and Methods

2.1. Plant Materials

*Arabidopsis thaliana* accession Columbia (Col-0) is the wild-type plant used throughout this research. The *Arabidopsis* ecotype Wassilewskija (WS) was used in this study to generate mapping populations. Mutants used were either T-DNA insertion lines, point mutations generated by the mutagen ethyl methanesulfonate (EMS), or a frame shift. Mutants used throughout these studies had been backcrossed to wild type one or more times.

2.1.a. T-DNA mutants

Insertional mutants contain a T-DNA inserted into the desired gene or promoter and were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University.

*ibr3-4* (SALK_004657; AT3G06810) and *ibr3-9* (SALK_033467; AT3G06810) were previously characterized (Zolman et al. 2007). *ibr10-1* contains a frameshift deletion discovered by screening a pool of T-DNA mutants (Alonso et al. 2003) and was previously characterized (Zolman et al. 2008). *aae18* (SALK_002180; AT1G55320) was previously described (Koo et al. 2006).

Mutant LONG CHAIN ACYL-COA SYNTHETASES (LACS) were studied for resistance to IBA in Chapter 3. *lacs4-1* (SALK_101543) and *lacs4-7* (SALK_120357) are both T-DNA insertions in AT4G23850. *lacs4-1* was previously described (Jessen et al. 2011; Jessen et al. 2015; Zhao et al. 2019). *lacs4-7* was characterized in this study. *lacs1-2* (SALK_138782; AT2G47240), *lacs2-3* (GABI_368C02; AT1G49430), *lacs3-1*
(SALK_027707; AT1G64400), lacs6 (SALK_069510c; AT3G05970), lacs7
(SALK_146444; AT5G27600), lacs8 (SALK_105118; AT2G04350), and lacs9-4
(SALK_124615; AT1G77590) were characterized for resistance to IBA in this study.

Mutant acyl-CoA oxidases were further studied in Chapter 5. acx1-2
(SALK_041464; AT4G16760), acx2-1 (SALK_006486; AT5G65110), acx3-6 (SALK_
044956; AT1G06290), acx4-3 (SALK_065013; AT3G51840), and acx5-1
(SALK_009998; AT2G35690) were previously described (Zolman et al. 2008; Rafeiza et
al. 2012).

2.1.b. Point mutants

Point mutations used in this study have an EMS generated nucleotide substitution
that results in a missense mutation or frameshift mutation. ibr3-1 (AT3G06810) and ibr1-
1 (AT4G05530) were previously characterized (Zolman et al. 2007; Zolman et al. 2008).

aux1-7 (AT2G38120) is a point mutation that was previously characterized (Pickett et al.
1990).

New enhancer mutants pulled from the forward genetic screen, described in
Chapter 4, will remain named hypocotyl resistance to IBA (HRI) and a corresponding
number until the causative mutation is identified.

Point mutants successfully identified and described in this study include lacs4-8,
amx3-7, and amx3-8. lacs4-8 (AT4G23850) is a G to C substitution at residue 1400
resulting in a conversion of an alanine to a valine and was characterized in Chapter 3.
amx3-7 (AT1G06290) is a G to A point mutation at nucleotide 336 that changes a
 glutamic acid to lysine at residue 137. amx3-8 is a C to T mutation at position 2204 that
caused a nonsense mutation at residue 382. \textit{acx3-7} and \textit{acx3-8} are described and characterized in Chapter 5.

2.1.c. Transgenic \textit{Arabidopsis} lines

New \textit{Arabidopsis} lines were generated to harbor a transgene of interest. The desired gene was amplified with PCR using wild type cDNA as a template with gene specific primers. Promoter sequences of interest were PCR amplified from wild-type DNA using primers specific for the entire intergenic sequence between the target gene and the upstream gene. All PCR products were electrophoresed on a 1\% agarose gel, excised, and cleaned up with a PCR clean up kit (IBI PCR/Gel Clean Up Kit). PCR products were digested with the appropriate enzymes when necessary and ligated into the designated vector backbone. The final construct was sequenced for verification.

Sequenced confirmed vectors containing the construct of interest were first transformed into \textit{Agrobacterium tumefaciens} and then into \textit{Arabidopsis} via the floral dip method (Clough and Bent 1998). Successfully transformed seedlings were selected by growing seedlings on 10 µg/mL Basta for 14 days. Homozygous lines were identified by screening T\textsubscript{3} on Basta.

All constructs were genotyped to confirm their presence in the final destination organism: plant or bacteria. Primer pairs to amplify the target sequence and enzymes required are listed in Table 2-1. PCR programs to create the constructs are listed in Table 2-2.

2.1.c.i. LACS4
LACS4 was amplified from a cDNA template made from wild-type RNA with LACS4Topo-F and LACS4Topo-R and cloned into the Gateway entry vector pcr8/TOPO. The stop codon of LACS4 was replaced with a six amino acid glycine-serine flexible linker with the Q5 Site-Directed Mutagenesis Kit (NEB) using the primers LACS4 c-term linker F and LACS4 c-term linker R. This LACS4 construct was moved into plant gateway expression vectors pEarlyGate101, pEarlyGate102 and pEarlyGate103 (Earley et al. 2006) with LR clonase (Thermo Fisher). pEarlyGate101:LACS4 and pEarlyGate103:LACS4 was transformed into wild type, lacs4-7, and lacs4-8.

2.1.c.ii. YFP-SRL and YFP-SRG

The plant expression vector UBQ10:YFP-GW (Michniewicz et al. 2015) was mutated independently by site-directed mutagenesis to end with SRL and SRG C-terminal tripeptides with the primers YFP-SRG F, YFP-SRG R, YFP-SRL F, and YFP-SRL R with the Quikchange XL Site-Directed Mutagenesis Kit (Agilent Technologies). UBQ10:YFP-SRL and UBQ10:YFP-SRG were transformed into wild-type Arabidopsis.

2.1.c.iii. LACS6

LACS6 was amplified from cDNA using the primers LACS6 TopoF and LACS6 TopoR using the PCR program Phusion IBR1-2. The LACS6 fragment was ligated into the Gateway entry vector pENTR. LACS6 was moved into the plant expression vector pEarlyGate100.

The proline at residue 497 was mutated to an alanine with the primers LACS6 P-A F and LACS6 P-A R with the Quikchange mutagenesis kit. Proline 497 was mutated to
a valine with the primers LACS6 P-V Fnew, and LACS6 P-V Rnew with the Phusion Site-Directed Mutagenesis Kit.

pEarlyGate100:LACS6, pEarlyGate100:LACS6 P497A, and pEarlyGate100:LACS6 P497V were transformed into wild type, lacs6, and lacs6lacs7.

2.1.c.iv. Promoter swapping constructs

The ACX3 promoter was amplified from wild type DNA using the primers ACX3upstF and ACX3upstR with the PhusionIBR1-2 PCR program. The ACX3 promoter fragment was digested with EcoR1-HF and Kpn1-HF and ligated into pBIN19. The IBR3 promoter was amplified with the primers IBR3upstF and IBR3newR using the PhusionIBR1-2 PCR program. The IBR3 promoter fragment was digested with EcoR1-HF and Kpn1-HF and ligated into pBIN19.

The ACX3 cDNA sequence was amplified from wild type cDNA with the primers ACX3cDNA3 and ACX3cDNA R using the PhusionIBR3 PCR program. The ACX3 cDNA fragment was digested with Sma1 and Sal1-HF and ligated into pBIN19:ACX3p and pBIN19:IBR3p to generate ACX3promoter:ACX3cDNA (Ap:Ac) and IBR3promoter:ACX3cDNA (Ip:Ac) respectively. IBR3 cDNA sequence was amplified from wild type cDNA using the primers IBR3cDNAF and IBR3cDNAR with the PhusionIBR3 PCR program. The IBR3 cDNA fragment was digested with Sma1 and Sal1 and ligated into pBIN19:ACX3p and pBIN19:IBR3p to generate ACX3promoter:IBR3cDNA (Ap:Ip) and IBR3promoter:IBR3cDNA (Ip:Ip) respectively.
2.1.c.v. PXN, PNC1, and ACH2

PXN was amplified from pUNI12863 using the primers pUNI F and pUNI R using the PhusionIBR3 PCR program. The PCR fragment was digested with Not1-HF and NheI-HF and ligated into 35SpBARN.

PNC1 was amplified from pUNI60372 using the primers pUNI F and pUNI R using the PhusionIBR3 PCR program. The PCR fragment was digested with Not1-HF and NheI-HF and ligated into 35SpBARN.

ACH2 was amplified with ACH2 Not1 and ACH2 Sma1 primers, digested with Sma1 and Not1-HF, and ligated into 35SpBARN.

Table 2-1. Primer sequences to create new constructs

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH2 cDNA</td>
<td>ACH2 Not1</td>
<td>CAATTCTAGCGGGCCGCCCATGAGGCCCTC</td>
</tr>
<tr>
<td></td>
<td>ACH2 Sma1</td>
<td>CCGCCCGGCGAGAAGGAGATATAACC</td>
</tr>
<tr>
<td>ACX3 cDNA</td>
<td>ACX3cDNA3</td>
<td>GAATCCCGGGAAAATGTCGGAATACCGATCC</td>
</tr>
<tr>
<td></td>
<td>ACX3cDNA R</td>
<td>TACTAGGTGACACATAAAGAGACAAAGCC</td>
</tr>
<tr>
<td>ACX3 promoter</td>
<td>ACX3upstF</td>
<td>CTGAATTCATGTCCCTAGTAAGG</td>
</tr>
<tr>
<td></td>
<td>ACX3upstR</td>
<td>ATGGTACCGGATTCGATACCGATTTTCC</td>
</tr>
<tr>
<td>IBR3 cDNA</td>
<td>IBR3cDNAAF</td>
<td>TGGGTACCTGGCCGGATCC</td>
</tr>
<tr>
<td></td>
<td>IBR3cDNAR</td>
<td>CGTGGGTGACACCCTCTAGAAGCTCTAGGTTAC</td>
</tr>
<tr>
<td>IBR3 promoter</td>
<td>IBR3upstF</td>
<td>GAGAATTCGGAGGTGAGGTTCTCTGTTTTC</td>
</tr>
<tr>
<td></td>
<td>IBR3upstR</td>
<td>GAGGTACCTCTACCTCTATACCTCTCGTC</td>
</tr>
<tr>
<td>LACS4 cDNA</td>
<td>LACS4BamHI</td>
<td>TCGGATACCCCTACCTCTGAGAAATTT</td>
</tr>
<tr>
<td></td>
<td>LACS4SalI</td>
<td>GTCGACATTCGAGCGAAGAAATAC</td>
</tr>
<tr>
<td>LACS4 cDNA</td>
<td>LACS4Topo-F</td>
<td>CACCATGTCGACAGAACAAATACATCTCTCC</td>
</tr>
<tr>
<td></td>
<td>LACS4Topo-R</td>
<td>CTACCCTCTGAAAGCAAAATTTTGTATTATAG</td>
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<tr>
<td>Glycine serine linker</td>
<td>LACS4cterm linker F</td>
<td>AGCGGCACAGGCGAATTGACACACGC</td>
</tr>
<tr>
<td></td>
<td>LACS4cterm linker R</td>
<td>GCCGAGCCGTTACCCTTGGAGAAATTTTG</td>
</tr>
<tr>
<td>LACS6 cDNA</td>
<td>LACS6 Topo F</td>
<td>CACCATGGATCTCTCTCTCTGCTCTTCC</td>
</tr>
<tr>
<td></td>
<td>LACS6 Topo R</td>
<td>TCACAAAATCCTATTAGCAGAAGGATCAG</td>
</tr>
<tr>
<td>LACS6 P497V</td>
<td>LACS6 P-V Fnew</td>
<td>AGCCCCATGTGGAGGGGAATAATGTG</td>
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<tr>
<td></td>
<td>LACS6 P-V Rnew</td>
<td>GATCCGCTGATGTATATGTCATTTCTGAGG</td>
</tr>
<tr>
<td>LACS6 P497A</td>
<td>LACS6 P-A F</td>
<td>CGCCACGGGACATGGGGCTGATCCG</td>
</tr>
<tr>
<td></td>
<td>LACS6 P-A R</td>
<td>CGGATCAGCCCGCATGCGGCGGCGG</td>
</tr>
<tr>
<td></td>
<td>pUNI-51 F</td>
<td>CGAAGTTATCTGGAATGCGGCCGCAAGG</td>
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<td>-------------------------------</td>
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<tr>
<td></td>
<td>pUNI-51 R</td>
<td>GTGACCGCTCAATGCTAGCGCAGGG</td>
</tr>
<tr>
<td>YFP-SRG</td>
<td>YFP-SRG F</td>
<td>GCAGAATTGAGCGCTGAGCTAGCTACCCCT</td>
</tr>
<tr>
<td></td>
<td>YFP-SRG R</td>
<td>CGACCGCTGACGTCAAAGCTAGAAGG</td>
</tr>
<tr>
<td>YFP-SRL</td>
<td>YFP-SRL F</td>
<td>GTCGAGCTGACGCTGACGCTAGAAGG</td>
</tr>
<tr>
<td></td>
<td>YFP-SRL R</td>
<td>GCTCAAGCGCTTCGACGCTTCAGAAGG</td>
</tr>
</tbody>
</table>

**Table 2-2. PCR programs to create constructs**

<table>
<thead>
<tr>
<th>Program</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp °C</td>
<td>Time</td>
<td>Temp °C</td>
<td>Time</td>
</tr>
<tr>
<td>PhusionIBR1-2</td>
<td>98</td>
<td>10 s</td>
<td>55</td>
<td>30 s</td>
</tr>
<tr>
<td>PhusionIBR3</td>
<td>98</td>
<td>10 s</td>
<td>62</td>
<td>30 s</td>
</tr>
</tbody>
</table>

2.1.d. Crosses to generate new lines

Double mutants were generated by crossing single mutants together and are listed in Table 2-3.

**Table 2-3. Crosses to generate double mutants**

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Mutant generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacs4-1 X ibr3-4</td>
<td>lacs4-1 ibr3-4</td>
</tr>
<tr>
<td>lacs4-7 X ibr3-4</td>
<td>lacs4-7 ibr3-4</td>
</tr>
<tr>
<td>lacs4-7 X ibr1-1</td>
<td>lacs4-7 ibr1-1</td>
</tr>
<tr>
<td>lacs4-7 X acx3-6</td>
<td>lacs4-7 acx3-6</td>
</tr>
<tr>
<td>lacs4-7 X lacs6</td>
<td>lacs4-7 lacs6</td>
</tr>
<tr>
<td>lacs4-7 X lacs7</td>
<td>lacs4-7 lacs7</td>
</tr>
<tr>
<td>lacs6 X lacs7</td>
<td>lacs6 lacs7</td>
</tr>
<tr>
<td>ibr3-9 X acx3-6</td>
<td>ibr3-9 acx3-6</td>
</tr>
<tr>
<td>ibr1-1 X acx3-6</td>
<td>ibr1-1 acx3-6</td>
</tr>
<tr>
<td>ibr10-1 X acx3-6</td>
<td>ibr10-1 acx3-6</td>
</tr>
<tr>
<td>ibr3-1 X acx1-2</td>
<td>ibr3-1 acx1-2</td>
</tr>
<tr>
<td>ibr3-1 X acx4-3</td>
<td>ibr3-1 acx4-3</td>
</tr>
<tr>
<td>ibr3-1 X acx5</td>
<td>ibr3-1 acx5</td>
</tr>
</tbody>
</table>
The DR5:GUS reporter construct was crossed into several mutants to evaluate auxin signaling. These crosses are listed in Table 2-4.

Table 2-4. Crosses to generate DR5:GUS reporter lines

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Line generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacs4-8 X DR5:GUS</td>
<td>lacs4-8 DR5:GUS</td>
</tr>
<tr>
<td>lacs4-8 ibr3-1 X DR5:GUS</td>
<td>lacs4-8 ibr3-1 DR5:GUS</td>
</tr>
<tr>
<td></td>
<td>ibr3-1 DR5:GUS</td>
</tr>
</tbody>
</table>

Enhancer mutants were outcrossed to WS to generate a mapping population in order to determine the genomic location of the causative mutation. Lines crossed to WS are listed in Table 2-5.

Table 2-5. Outcrosses to WS to generate mapping populations

<table>
<thead>
<tr>
<th>Cross</th>
<th>Mapping population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z377 ibr3-1 X WS</td>
<td>Z377 ibr3-1 Col/WS F₂</td>
</tr>
<tr>
<td>HRI#46 ibr3-1 X WS</td>
<td>HRI#46 ibr3-1 Col/WS F₂</td>
</tr>
<tr>
<td>HRI#70 ibr3-1 X WS</td>
<td>HRI#70 ibr3-1 Col/WS F₂</td>
</tr>
<tr>
<td>HRI#11 ibr1-1 X WS</td>
<td>HRI#11 ibr1-1 Col/WS F₂</td>
</tr>
<tr>
<td>HRI#41 ibr1-1 X WS</td>
<td>HRI#41 ibr1-1 Col/WS F₂</td>
</tr>
</tbody>
</table>

2.1.e. Genotyping

All mutant plants and constructs were genotyped for confirmation. T-DNA insertion mutants require two primer sets to confirm the mutation. The first primer pairs are located outside of the known location of the T-DNA insertion site. These primers will only generate a product if the T-DNA is not present. The second primer set is designed to confirm the presence of the T-DNA using a primer located in the genomic region outside of the T-DNA insertion site and a primer located within the T-DNA sequence. This
primer pair will only generate a product if the T-DNA is present. Primer pairs to genotype T-DNA mutants are listed in Table 2-6.

Point mutations are genotyped with dCAPS primers. Primers are specifically designed to recognize the sequence next to the genomic point mutation, but will introduce a restriction endonuclease site (Neff et al. 2002). The point mutation will either create or destroy the enzyme site. This PCR product can be digested using the appropriate restriction enzyme to distinguish the wild type product from the mutant product. Primer pairs and the enzyme required for genotyping are listed in Table 2-7.

Transgenic plants were genotyped using primers specific for the transgene that can distinguish the transgenic copy versus the endogenous copy.

All primer sequences used to genotype mutant plants are listed in the 5’ to 3’ direction in Table 2-8.

**Table 2-6. Primers pairs to genotype T-DNA mutants**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Pair</th>
</tr>
</thead>
</table>
| *lacs4-1* | LACS4-1 & LACS4-3  
|  
|  | LACS4-6 & LB-SALK |
| *lacs4-7* | LACS4-5 & LACS4-7  
|  
|  | LACS4-7 & LB-SALK |
| *lacs6-1* | LACS6-1 & LACS6-4  
|  
|  | LACS6-4 & LB-SALK |
| *lacs7-1* | LACS7-3 & LACS7-5  
|  
|  | LACS7-5 & LB-SALK |
| *lacs1-2* | LACS1-6 & LACS1-7  
|  
|  | LACS1-7 & LB-SALK |
| *lacs2-3* | LACS2-3 & LACS2-4  
|  
|  | LACS2-4 & LB-GABI |
| *lacs3-1* | LACS3-1 & LACS3-2  
|  
|  | LACS3-2 & LB-SALK |
| *lacs8-2* | LACS8-6 & LACS8-7  
|  
|  | LACS8-7 & LB-SALK |
| *lacs9-4* | LACS9-3 & LACS9-4  
<p>|<br />
|  | LACS9-4 &amp; LB-SALK |
| <em>acx1-2</em> | ACX1-20 &amp; ACX1-21 |</p>
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Pair</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>acx2</td>
<td>ACX2-2 &amp; ACX2-12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACX2-7 &amp; LB-SALK</td>
<td></td>
</tr>
<tr>
<td>acx3-6</td>
<td>ACX3-27 &amp; ACX3-28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACX3-28 &amp; LB-SALK</td>
<td></td>
</tr>
<tr>
<td>acx4-3</td>
<td>ACX4-10 &amp; ACX4-11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACX4-11 &amp; LB-SALK</td>
<td></td>
</tr>
<tr>
<td>acx5</td>
<td>ACX5\textsubscript{New}8 &amp; ACX5\textsubscript{New}9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACX5\textsubscript{New}9 &amp; LB-SALK</td>
<td></td>
</tr>
<tr>
<td>ibr3-4</td>
<td>F3E22-30 &amp; F3E22-31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3E22-31 &amp; LB-SALK</td>
<td></td>
</tr>
<tr>
<td>ibr3-9</td>
<td>F3E22-21 &amp; F3E22-18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3E22-18 &amp; LB-SALK</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-7. Primer pairs to genotype point mutations**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Pair</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>ibr3-1</td>
<td>F3E22-28 &amp; F3E22-43</td>
<td>EcoNI</td>
</tr>
<tr>
<td>ibr1-1</td>
<td>T1J24-1 &amp; T1J24-11</td>
<td>PstI-HF</td>
</tr>
<tr>
<td>ibr10-1</td>
<td>AT4G14420-1 &amp; AT4G14420-2</td>
<td>HindIII</td>
</tr>
<tr>
<td>lacs4-8</td>
<td>LACS4-1 &amp; LACS4-3</td>
<td>PstI-HF</td>
</tr>
<tr>
<td>acx3-7</td>
<td>ACX3-32 &amp; ACX3-35</td>
<td>XhoI</td>
</tr>
<tr>
<td>acx3-8</td>
<td>ACX3-37 &amp; ACX3-27</td>
<td>NdeI</td>
</tr>
</tbody>
</table>

**Table 2-8. Primer pairs to genotype transgenic lines**

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Primer Pairs</th>
<th>Selectable Marker</th>
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</thead>
<tbody>
<tr>
<td>DR5:GUS</td>
<td>DR5 &amp; Gus-2</td>
<td>Basta</td>
</tr>
<tr>
<td>pEarlyGate101:LACS4</td>
<td>35S-F3 &amp; LACS4-7</td>
<td>Basta</td>
</tr>
<tr>
<td>pEarlyGate102:LACS4</td>
<td>35S-F3 &amp; LACS4-7</td>
<td>Basta</td>
</tr>
<tr>
<td>pEarlyGate103:LACS4</td>
<td>35S-F3 &amp; LACS4-7</td>
<td>Basta</td>
</tr>
<tr>
<td>UBQ10:YFP-LACS4</td>
<td>YFP-F &amp; LACS4-7</td>
<td>Basta</td>
</tr>
<tr>
<td>pEG100:LACS6</td>
<td>35S-F3 &amp;</td>
<td>Basta</td>
</tr>
<tr>
<td>pEG100:LACS6 P-A</td>
<td>35S-F3 &amp;</td>
<td>Basta</td>
</tr>
<tr>
<td>pEG100:LACS6 P-V</td>
<td>35S-F3 &amp;</td>
<td>Basta</td>
</tr>
<tr>
<td>Ap:Ac</td>
<td>pBIN-F &amp; ACX3-38</td>
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</tr>
<tr>
<td></td>
<td>ACX3-37 &amp; pBIN-R</td>
<td></td>
</tr>
<tr>
<td>Ap:Ic</td>
<td>ACX3-1 &amp; F3E22-51</td>
<td>Basta</td>
</tr>
<tr>
<td>Ip:Ic</td>
<td>pBIN F &amp;</td>
<td>Basta</td>
</tr>
<tr>
<td></td>
<td>FE322-11 &amp; pBIN-R</td>
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</tr>
<tr>
<td>Ip:Ac</td>
<td>F3E22-18 &amp; ACX3-38</td>
<td>Basta</td>
</tr>
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<td>35S:ACX3</td>
<td>35S-F3 &amp;</td>
<td>Basta</td>
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<tr>
<td>Primer name</td>
<td>Sequence 5’ - 3’</td>
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<td>------------</td>
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<td>ACH2-1</td>
<td>CGATATCCGATTGCCAGATGGAATTAGTATC</td>
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<td>ACX1-20</td>
<td>TTCAGGCCTATTGGTTAGGTGCATTC</td>
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<td>ACX1-21</td>
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<td>ACX2-2</td>
<td>AATCAAAATCCGAGACTAGATGTAGAATC</td>
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<td>ACX2-7</td>
<td>CAACGATTGTCTTACCTACTATC</td>
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<td>ACX2-12</td>
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<td>ACX3-27</td>
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<td>ACTACATTGATCTGACAGGTACAG</td>
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<td>ACX3-1</td>
<td>CTAAGGAGAGGTTCATGTACTGGAT</td>
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<td>ACX3-7</td>
<td>AACAAACTTCTCAAGGACTGGAGTGGAG</td>
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<td>ACX3-32</td>
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<tr>
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Table 2-9. Primer sequences for genotyping primers
PCR was used to amplify target regions for genotyping. The PCR program SSLP was used for genotyping when the amplified product is less than 500 base pairs. When the desired PCR product is between 500 and 1000 base pairs, the PCR program 55-1 was used. The SSLP and 55-1 programs are listed Table 2-10.

Table 2-10. PCR programs for genotyping

<table>
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<td>Time</td>
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<td>55-1</td>
<td>95</td>
<td>30 s</td>
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LACS3-1  CGCCTGTATTTAGTGTTAGAATCCTCC
LACS3-2  CATTTTCAGGCTTGCAATGCTCATTG
LACS4-1  GTCTTTCGCAGTGGCGATTTTCACCACCT
LACS4-3  GTCCTACATCTCATTTTTACCTACCTAGAATG
LACS4-5  GCATTATGGGGCTTATATGATGATACG
LACS4-6  GAGTTGGAGACTGAAAAAGACCTACCCG
LACS4-7  CATCGCCGAAAATCCATATCCCTTGCGATAG
LACS6-1  CTTGTCTCTGTAGCAATGGCTGTTGCTG
LACS6-4  CAGCATTGCTACATAAGCCTAAAAAACAG
LACS7-3  GTACAAGGTTCCGATGGAACCTATTG
LACS7-5  GCATGATTCACAAACCACCTGACTCTGCTG
LACS8-6  GAGAATTGTGTTAGGTAACAGTGTAAC
LACS8-7  CACCTGTGAAATTGTGATCCGCAAGAATG
LACS9-3  TATCTCCAGCAACCAAGATG
LACS9-4  AAGCTTCCGGAAGAATTTG
LB-SALK  CAAACCAGGCGGCTTGCTGCAACTC
LB-GABI  ATATTGACCATCATACTCAGTC
pBIN F   CTCGAAGGCGATTAAGTGGGTAACGCCAG
pBIN R   CTTATGCTTTCGGCTGATTTGGTTGG
PNC1-1   GCACTCTTGATGAAAGCCTAGTGAGGCCGC
PXN-1    CATTAAACGGTACCCGCAAGGCAGCCAC
T1J24-1  CGCAACTCATTTCATCGCTTCTG
T1J24-11 TGAAAGTGAGAGATGGTGAAACCTGTCTGCG

**PCR** was used to amplify target regions for genotyping. The PCR program SSLP was used for genotyping when the amplified product is less than 500 base pairs. When the desired PCR product is between 500 and 1000 base pairs, the PCR program 55-1 was used. The SSLP and 55-1 programs are listed Table 2-10.
2.1.f. Transient expression in *Nicotiana benthamiana*

*Nicotiana benthamiana* was used for transient expression of *LACS4*. *N. benthamiana* was grown for 4-8 weeks at 22 °C until the plants had 4-8 leaves. pEarlyGate102:LACS4 and UBQ10:YFP-SRL in *A. tumefaciens* were grown overnight at 30 °C. Cultures were incubated in infiltration media (10 mM MgCl₂, 10 mM MES, and 200 µM acetosyringone) and gently rocked at room temperature for 4 hours. The cells were resuspended in fresh infiltration media to an OD₆₀₀ of 1. Cultures of each construct were mixed at a ratio of 1:1 and infiltrated into young tobacco leaves of plants with 4-8 leaves using a 1 mL needleless syringe. Leaves were imaged 48 hours after infiltration.

2.2. Phenotypic Assays

Seeds were surface sterilized with 30 % bleach and 0.1 % triton for 10 minutes, rinsed three times with sterile H₂O, suspended in 0.1 % agar, and imbibed at 4 °C for 3-5 days. Seeds were grown on plant nutrient media (PN) (Haughn and Somerville 1986) supplemented with hormone or 0.5 % sucrose (PNS) as indicated. Plates were sealed with micropore surgical tape, incubated at 22 °C under continuous yellow light if supplemented with hormone or white light if the media contained no additives. Seedlings were grown for the specified number of days. After phenotypic assays were completed, seedlings were transferred to soil and grown at 22 °C under continuous light until senescence.
2.2.a. Primary root elongation

Primary root length was measured after 7 days of growth on indicated media. Root length was measured in millimeters (mm).

2.2.b. Lateral root initiation

Lateral root density was determined by growing seeds on PN for 4 days, transferring seedlings to PN or PN + IBA plates and growing for an additional 4 days. The number of lateral roots were counted with a Leica Zoom 2000. Lateral root density is calculated as the number of lateral roots per mm of primary root length.

2.2.c. Root hair elongation

Seedlings were grown on the indicated media for 8 days. Seedlings were immersed in 5 % glycerol and imaged with Evos XL Core (Thermo Fisher) microscope on the 4 X objective. Root hair length was measured with ImageJ software (Schneider et al. 2012).

2.2.d. Dark grown hypocotyl elongation.

To assay IBA effects on hypocotyl length, seeds were plated on media with the indicated concentration of IBA. Plates were incubated under yellow light for 1 day and in darkness for an additional 5 days before measuring hypocotyl length. Sucrose dependence was determined by plating seeds on PN and PNS. Plates were incubated under light for 1 day. Plates were then incubated in darkness for an additional 5 days before measuring hypocotyl length in mm.
2.2.e. Gus staining

β-glucuronidase (GUS) was used as a reporter to visualize the location of gene expression or auxin signaling in plant tissue. Genotypes containing the DR5:GUS transgene were grown on PN plates for 5 days then transferred to liquid PN without hormone or containing 50 µM IBA, or 1 µM IAA and gently rocked at room temperature for 2 hours. GUS was visualized by incubating seedlings in 100 mM NaPO₄ pH 7.0, 0.5 mM K₃[Fe(CN₆)], 0.5 mM K₄[Fe(CN₆)] 3H₂O, 0.01 % Triton X-100, and 0.5 mg/mL X-Gluc for 3 hours at 37 °C, followed by washes with 50 % EtOH, 75 % EtOH, and 95 % EtOH. Plants were stored in 50 % glycerol solution at 4 °C and imaged with Evos XL Core (Thermo Fisher) on 20 X and 40 X objectives.

2.2.f. Root phenotypes in phosphate starvation conditions.

Phosphate starvation conditions were created by replacing the 250 mM KP0₄ in PN with equimolar amount of KCl, similar to Williamson et al. 2001, so that the volume remained the same. Phosphate starvation conditions used were 0.1 mM KP0₄, 0.05 mM KP0₄, or 0 mM KP0₄ and were compared to a typical phosphate concentration of 2.5 mM KP0₄.

To calculate lateral root density, plants were grown on the indicated phosphate conditions for 12 days. Lateral root density was quantified as the number of lateral roots per primarily root length.

Root hair elongation was measured by growing plants on the indicated concentration of phosphate for 8 days. Root hairs were imaged with the Evos XL Core (Thermo Fisher) microscope at 4 X. Images were captured approximately in the middle of the root to ensure the root hairs were mature and made contact with the media. The
length of 20 root hairs were measured with ImageJ for each plant. Average root hair length of each plant was used to calculate overall average root hair length and perform statistical analysis.

2.3. Mutagenesis and mutant isolation

*ibr3-1* and *ibr1-1* seeds were soaked in 0.24 % ethyl methanesulfonate (EMS) for 18 hours in the dark, washed extensively, and transferred to soil to allow M2 seeds to set.

2.3.a. Enhancer mutant isolation

Approximately, 15,000 mutagenized M2 *ibr3-1* and *ibr1-1* seeds were plated on 60 µM IBA. These seeds were grown for 1 day under yellow light, then transferred to darkness and grown for an additional 5 days and screened for elongated hypocotyls compared to the *ibr3-1* or *ibr1-1* parental lines. Mutants recovered from the enhanced hypocotyl resistance to IBA screen hypocotyls were named hypocotyl resistance to IBA (HRI) followed by a number. Putative hypocotyl IBA resistant enhancers were recovered on PN for 5 days before transferring to soil. M3 progeny were retested to validate the mutant phenotype in IBA response assays of root elongation. Six enhancer mutants in *ibr3-1* background and five enhancer mutants in *ibr1-1* background were retained for further characterization.

2.3.b. Single mutant isolation

Enhancer double mutants were backcrossed to wild type and appropriate mutant parent (*ibr3-1* or *ibr1-1*). F2 plants backcrossed to wild type were grown on 10 µM IBA and screened for both seedlings with an intermediate IBA resistant root phenotype and an enhanced resistance to IBA. Enhancers are presumed to be the double mutant. F2 plants
backcrossed to wild type that genotyped wild-type at ibr3-1 or ibr1-1 are presumed to be the new mutation separated from the ibr3-1 or ibr1-1 parent.

2.4. Whole genome sequencing

Ten independently isolated Z377 ibr3-1 lines from a backcross to ibr3-1 were selected and pooled for sequencing in parallel with wild type and ibr3-1. DNA was extracted from approximately 2,000 seedlings grown on filter paper for 10 days. Z377ibr3-1, HRI#41 ibr1-1, HRI#70 ibr3-1, and HRI#11 ibr1-1 were sequenced at the Genome Technology Access Center at Washington University in St. Louis on an Illumina-HiSeq2000. Gene candidates were first narrowed down by identifying homozygous single nucleotide polymorphisms (SNPs) consistent with EMS mutagenesis in protein coding regions. Mutations common between Z377 ibr3-1 and wild type or ibr3-1 were eliminated, leaving mutations unique to Z377 ibr3-1.

dCAPS primers were designed to genotype the point mutation in lacs4-8. LACS4-1 and LACS4-3 were used to amplify the region spanning the lacs4-8 mutation. The PCR product was digested with Pst1-HF and run on a 3% agarose gel. The digest will cut the wild type sequence but not the mutant.

2.5. Confocal microscopy

Confocal images were gathered with a Zeiss LSM 700 laser scanning confocal microscope using the 20 X lens. pEarlyGate103:LACS4 was imaged in 7-day-old Arabidopsis root hairs of T2s showing rescued IBA resistant phenotypes when grown on 15 µM IBA. 7-day-old wild-type Arabidopsis seedlings were stained with 5 µM 8-(4-Nitrophenyl) Bodipy (Toronto Research Chemicals) for 10 min and excited with a 488
nm laser. UBQ10:YFP-SRL and UBQ10:YFP:SRG were viewed in both roots and cotyledons of 7-day-old *Arabidopsis* seedlings. Localization of pEarlyGate102:LACS4 and UBQ10:YFP-SRL was imaged 48 hours after infiltration into *N. benthamiana* leaves.

### 2.6. RNA extraction and expression quantification

Seedlings were grown on filter paper under white light for 5 days. RNA was extracted by grinding seedlings in liquid nitrogen and using the IBI Total RNA Mini Kit (Plant; MidSci). cDNA was synthesized using ProtoScript II Reverse Transcriptase (NEB). Gene expression was determined by qRT-PCR using Bullseye EvaGreen qPCR Mix (MidSci) on a BioRad CFX96 Real Time PCR System. Target gene expression was normalized to UBIQUITIN 10 and calculated with the ΔΔCT method. Primers were designed with QuantPrime (Arvidsson et al. 2008) and are listed in Table 2-11. No reverse transcriptase reactions were used as a control. Experiments were performed with both biological and technical triplicates.

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2.7. Expression and purification of recombinant LACS4

LACS4 cDNA was amplified with primers LACS4Sal1 and LACS4BamHI, digested with Sal1-HF and BamHI-HF, and ligated into the pZA31 expression vector which adds an N-terminal HN tag and controls expression through the TetR system (Lutz and Bujard 1997). Primer sequences are listed in Table 2-1. pZA31:LACS4 was transformed into Escherichia coli DH5αZ1, a strain modified to constitutively express TetR from the chromosome for bacterial expression (Lutz and Bujard 1997).

pZA31:LACS4 cultures were grown with 25 µg/mL chloramphenicol at 37 °C to an OD600 of 0.5, induced with 200 µg/mL anhydrotetracycline, then grown overnight at 16 °C. Cells were pelleted, resuspended in 50 mM Tris pH 7.5, 300 mM NaCl, 0.1 % Triton X-100 and Pierce protease inhibitor mini tablets (Thermo Fisher). Cells were lysed by incubating with 2 mg/mL lysozyme at 4 °C for 1 hour followed by sonication with ten 30 second pulses. Cell debris was removed by centrifuging for 30 minutes at 20,000 g at 4 °C. Supernatant was then passed through a 0.22 µM filter to remove remaining cells and debris.

The filtered supernatant was passed through an equilibrated 5 mL cobalt column on an ÄKTA prime FPLC (GE Healthcare). LACS4 recombinant protein was eluted in 1 mL fractions with 50 mM Tris pH 7.5, 300 mM NaCl, with increasing imidazole gradient up to 200 mM. Eluted fractions containing LACS4 were pooled and dialysed overnight at 4 °C in 10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, and 40 % glycerol. Desalting
buffer was changed and allowed to continue to desalt for an additional 4 hours. Protein concentration was determined by Bradford assay. LACS4 was aliquoted and stored at -80 °C.

2.8. *in vitro* assays for enzyme activity

2.8.a. CoA synthetase activity

LACS4 CoA synthetase activity was assayed by measuring pyrophosphate (PPI) production. 1 µg of LACS4 was incubated in a 50 µL reaction of 0.5 mM CoA, 45 mM ATP, 0.1 % Triton X-100, 1 mM DTT, 100 mM Tris pH 7.5, and the indicated substrate for 10 minutes at 24 °C. 10 mM stocks of IBA potassium salt (Chem Cruz), IAA sodium salt (Cayman Chemical Co), IAA-alanine (Milipore-Sigma), methyl-IAA (Milipore-Sigma), and tryptophan dissolved in 50 % EtOH were tested as substrates. LACS4 incubated with 5 % EtOH served as the negative control. PPI was measured with a Pyrophosphate Assay Kit (Milipore-Sigma) according to instructions by adding 50 µL of reaction buffer to each CoA synthetase reaction and incubating 15 additional minutes at 24 °C. PPI fluorescence was measured with λex=316 nm λem= 456 nm on a Biotek Cytation 3.

2.8.b. Acyl-CoA oxidase activity

Seedlings were grown on filter paper for 4 days under white light. Approximately 700 mg of tissue was ground to a fine powder in liquid nitrogen. The ground tissue was resuspended in 1.8 mL extraction buffer made of 50 mM KPO4 pH 7.6, 50 µM FAD, 0.01 % Triton X-100, 10 uL protease inhibitor cocktail, and 100 µg/mL BSA and incubated at 4 °C for 10 minutes. The plant extracts were passed over PD-10 desalting columns (GE
Healthcare) equilibrated with extraction buffer. Protein was eluted in 3.5 mL extraction buffer. Protein concentration was determined by the Bradford assay. All extracts were diluted to 500 µg/mL.

Acyl-CoA oxidase activity was assayed by incubating 200 µL of the crude protein extracts, 200 µL reaction buffer, and the indicated substrate. Oxidation of the substrate generates H$_2$O$_2$, which is measured using a spectrophotometer. H$_2$O$_2$ production was measured over 10 minutes.
Chapter 3: LACS4

Z377 was identified as being defective in LACS4. This mutant was discovered in a screen for mutants with enhanced resistance to IBA in the ibr3-1 background. lacs4 strongly enhances the effects of ibr3-1 in IBA responses. A lacs4 single mutant is resistant to IBA effects in primary root and dark grown hypocotyl inhibition but retains the ability to develop some lateral roots although at a reduced density when compared to wild type. Here I present evidence that LACS4 is the CoA synthetase that catalyzes the addition of CoA onto IBA, the first step in its β-oxidation. Sections of this work have been accepted for publication as a research article in Plant Physiology titled LONG CHAIN ACYL COA SYNTHETASE 4 catalyzes the first step in peroxisomal indole-3-butyric acid to IAA conversion: DOI: 10.1093/plphys/kiaa002.

3.1. Z377 mutant phenotypes

IBA metabolism to IAA parallels fatty acid β-oxidation; enzymes with similar activities likely will be required for the analogous steps. It is hypothesized that IBA is charged with CoA by a CoA synthetase before β-oxidation can proceed. My identification of LACS4 in an unbiased screen for IBA resistance was exciting, as the CoA synthetase functioning on IBA had not yet been reported. It was necessary to characterize our Z377 mutant to tease apart its role in IBA metabolism, test its responses in common IBA responses, and determine if it is specific to IBA or has a general auxin defect.
3.1.a. Z377 ibr3-1 IBA phenotypes

Z377 ibr3-1 has increased resistance to IBA in root elongation (Figure 3-1). Wild-type plants grown on 20 µM IBA have a primary root length less than 20 % of that when grown without hormone (Figure 3-1 A and B). ibr3-1 is double that with a primary root length of 40 % without hormone. In stark contrast, Z377 ibr3-1 retains a root length of above 80 % on the same IBA concentration.

Z377 ibr3-1 is defective in lateral root initiation when stimulated with IBA, resembling wild type grown in the absence of hormone (Figure 3-1 C). This phenotype is similar to the ibr3-1 parent line.

Wild-type plants have reduced hypocotyl length when grown on IBA than media without hormone (Figure 3-1 D). The enhanced resistance of Z377 ibr3-1 to IBA extends to dark grown hypocotyl elongation.

3.1.b. Z377 IBA phenotypes

Separation of Z377 from the previously-characterized ibr3 revealed that Z377 has a notable IBA-resistance profile on its own. Z377 has IBA resistance in root elongation assays intermediate between wild-type seedlings and the Z377 ibr3-1 enhancer and were comparable to the single ibr3 mutation (Figure 3-1 A and 1B).

Surprisingly, Z377 remained sensitive to IBA induced lateral rooting (Figure 3-1 C). Z377 develops a similar mean number of lateral roots to wild type, with somewhat reduced lateral root density due to its longer primary root. This phenotype is distinct from ibr3, which has statistically similar lateral root density when grown with or without IBA.
(Figure 3-1 C) and other ibr mutants (Zolman et al. 2008). This demonstrates that the effects of ibr3 in lateral root initiation are epistatic to those of Z377.

Figure 3-1. Z377 IBA resistant phenotypes.
(A) Primary root elongation of 7-day-old seedlings grown on increasing concentrations of IBA (±SE, n ≥10).
(B) Images of 7-day-old seedlings grown on 20 μM IBA. Scale bar = 5 mm
(C) Lateral root density of 8-day-old seedlings was quantified by dividing the number of lateral roots by the primary root length. Statistical significance was determined by two-way ANOVA with post hoc Tukey HSD. Common letters indicate no significant difference (±SE, n ≥8, p <0.001).
(D) Length of dark grown hypocotyls. Seedlings were grown for 1 day in light and 5 days in darkness. Statistical significance was determined by two-tailed t-test versus Wt on the same condition (±SE, n ≥15, *p< 0.001).
Z377 is also resistant to IBA in dark grown hypocotyl elongation and is comparable to ibr3-1 (Figure 3-1 D), indicating the IBA response defects are not limited to specific tissues and effects extend throughout the plant.

3.1.c. Z377 and Z377 ibr3-1 responses to other hormones

To further understand the range of Z377-related phenotypes, I measured Z377 and Z377 ibr3-1 responses on other plant hormones that may influence auxin or IBA response.

3.1.c.i. Z377 and Z377 ibr3-1 on endogenous hormones

First, Z377 and Z377 ibr3-1 were tested for responses to IAA to determine if these mutants had IBA-specific defects or a generalized resistance to auxin. Z377 and Z377 ibr3-1 remained sensitive to inhibition of primary root elongation by IAA (Figure 3-2 A) suggesting that the mutation in Z377 is not involved in auxin sensing or auxin signaling.

Another plant hormone, ethylene, influences root development through interactions with auxin (Qin and Huang 2018). To determine if Z377 and Z377 ibr3-1 are defective in ethylene response, roots were measured after 7 days of growth on 1-Aminocyclopropane-1-carboxylic acid (ACC). ACC is direct precursor for plants to synthesize ethylene (Yang and Hoffman 1984) and is used as an indicator of ethylene responses. Z377 and Z377 ibr3-1 were inhibited similarly to wild type when grown on ACC (Figure 3-2 B), displaying no apparent defects in ethylene response or signaling.
3.1.c.ii. Z377 and Z377 ibr3-1 on synthetic auxins

To fully characterize Z377 and Z377 ibr3-1 on auxins commonly tested in literature, the mutants were evaluated for their response on the synthetic auxins 2-4-DB, 2-4-D, and 1-Naphthaleneacetic acid (NAA).

2-4-DB is a synthetic auxin structurally similar to 2-4-D but has its side chain elongated by two carbons, mimicking the structural difference between IAA and IBA. 2-4-DB is believed to be β-oxidized in a similar mechanism to that of IBA (Wain and Wightman 1954). Z377 is resistant to 2-4-DB and Z377 ibr3-1 has enhanced resistance (Figure 3-3 A). The ability of Z377 to enhance the effects of ibr3-1 extends to 2-4-DB. Resistance to 2-4-DB and IBA suggests that Z377 participates in β-oxidation reactions.

Z377 and Z377 ibr3-1 are sensitive to 2-4-D and NAA and are comparable to wild type (Figure 3-3 B and C). Sensitivity to these auxins provides additional evidence that Z377 has normal responses to auxin perception and signaling.

3.1.d. Z377 sucrose dependence

Enzymatic components of the IBA metabolic pathway discovered to date are contained within the peroxisome and several mutants with peroxisomal fatty acid β-oxidation defects also have IBA resistant phenotypes (Hayashi et al. 1998; Zolman et al. 2000; Adham et al. 2005). Plants with severe defects in fatty acid β-oxidation are compromised in their ability to generate the energy necessary for germination and growth before plants are photosynthetically capable. Defects in fatty acid β-oxidation can be exposed by examining dark grown hypocotyls on media without sucrose, which requires metabolism of seed storage lipids. Addition of sucrose to the media bypasses this requirement, allowing development to proceed. To examine if Z377 is involved in fatty
acid β-oxidation or peroxisomal functions, Z377 was tested for sucrose dependent growth. Both Z377 and Z377 ibr3-1 have comparable elongation of dark grown hypocotyls in media lacking or containing sucrose (Figure 3-4). This demonstrates that fatty acid β-oxidation is not compromised and the mutants have normally functioning peroxisomes.
Figure 3-2. Z377 and Z377 ibr3-1 have normal growth on IAA and ACC.

(A) Primary root length of 7-day-old seedlings grown on increasing concentrations of IAA (±SE, n≥8).
(B) Primary root length of 7-day-old grown on ACC. Statistical significance determined by two-tailed t-test between Wt on same treatment (±SE, n≥8, n=6 ibr3-1, p<0.05).
Figure 3-3. Z377 responses on synthetic auxins.

(A) Primary root length of 7-day-old grown on 2-4-DB. Statistical significance determined by two-tailed t-test between Wt on same treatment (±SE, n ≥9, p<0.05).
(B) Primary root length of 7-day-old grown on 2-4D. Statistical significance determined by two-tailed t-test between Wt on same treatment (±SE, n ≥9, p<0.05).
(C) Primary root length of 7-day-old grown on NAA. Statistical significance determined by two-tailed t-test between Wt on same treatment (±SE, n ≥15, p<0.05).
Figure 3-4. Z377 is not sucrose dependent.

Hypocotyl length of seedlings grown on media with and without sucrose for 1 day in light and 5 days in darkness. Statistical significance determined by one-tailed t-test between sucrose treatments (±SE, n ≥13, p <0.05).
3.2. Identification of Z377 as LACS4

Z377 ibr3-1 is a mutant pulled from a screen for enhanced resistance to IBA in primary root elongation. Rough mapping and whole genome sequencing was used to identify the causative mutation in Z377 ibr3-1.

3.2.a. Mapping

Z377 ibr3-1 was outcrossed to the A. thaliana ecotype Wassilewskija (WS). F2 seedlings that retained the enhanced IBA resistant phenotype were genotyped at several genomic markers. Markers tested and recombination frequencies are listed in Table 3-1. Z377 ibr3-1 demonstrated low recombination frequency at the top of chromosome 3, as expected due to the ibr3-1 mutation. Regions with lower recombination frequency than expected was the middle of chromosome 1, the middle of chromosome 4, and the bottom of chromosome 5.

3.2.b. Whole genome sequencing

15 mg of seeds from independent Z377 ibr3-1 and Z377 F3s were grown for 10 days. These seedlings were pooled together and DNA was extracted from the bulked seedlings. Gene candidates were first narrowed by identifying homozygous single nucleotide polymorphisms (SNPs) consistent with EMS mutagenesis in protein coding regions in Z377 ibr3-1. Mutations common between Z377 ibr3-1 and wild type or Z377 ibr3-1 and ibr3-1 were eliminated, leaving 10 mutations unique to Z377 ibr3-1. Nine candidate genes were clustered on chromosome 4 (Table 3-2). AT4G23850, which encodes LACS4, showed an alanine to valine substitution in the Z377 mutant (Figure 3-6 A) and became the prime candidate because LACS6 and LACS7 are known peroxisomal
enzymes responsible for priming fatty acids with CoA in the first step in fatty acid β-oxidation (Figure 1-1 and (Fulda et al. 2004). Other candidate genes did not have a clear link to peroxisomes, auxin responses, IBA metabolism, or lipid metabolism, further supporting the hypothesis that the mutation in LACS4 might be the causative mutation in Z377.

Z377 has the unusual ability to initiate some lateral roots when grown on IBA. However, the ACX family of genes were not candidates because all members were wild-type in the whole genome sequencing data.
### Z377 ibr3-1 mapping

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Marker</th>
<th>Recombination frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top chromosome 1</td>
<td>F19P19</td>
<td>57 %</td>
</tr>
<tr>
<td>Middle chromosome 1</td>
<td>T12C22</td>
<td>27 %</td>
</tr>
<tr>
<td>Middle chromosome 1</td>
<td>F3F19</td>
<td>59 %</td>
</tr>
<tr>
<td>Bottom chromosome 1</td>
<td>F5I14</td>
<td>51.7 %</td>
</tr>
<tr>
<td>Bottom chromosome 2</td>
<td>SNP1</td>
<td>48 %</td>
</tr>
<tr>
<td>Top chromosome 3</td>
<td>GAPC</td>
<td>3.8 %</td>
</tr>
<tr>
<td>Top chromosome 3</td>
<td>NGA172</td>
<td>0 %</td>
</tr>
<tr>
<td>Middle chromosome 3</td>
<td>T23J7</td>
<td>43 %</td>
</tr>
<tr>
<td>Middle chromosome 4</td>
<td>T5L19</td>
<td>30 %</td>
</tr>
<tr>
<td>Bottom chromosome 4</td>
<td>NGA1107#2</td>
<td>48 %</td>
</tr>
<tr>
<td>Top chromosome 5</td>
<td>MRN17</td>
<td>48 %</td>
</tr>
<tr>
<td>Middle chromosome 5</td>
<td>GA3</td>
<td>52 %</td>
</tr>
<tr>
<td>Bottom chromosome 5</td>
<td>MGI19</td>
<td>36.9 %</td>
</tr>
</tbody>
</table>

**Table 3-1. Rough mapping of Z377 ibr3-1.**

Location of the genomic markers tested and recombination frequency at each marker is included.
### Gene Candidates in Z377

<table>
<thead>
<tr>
<th>ATG number</th>
<th>Gene name and description</th>
<th>Mutation tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT4G15280</td>
<td>UDP-GLUCOSYL TRANSFERASE 71B5</td>
<td>GK-485D08</td>
</tr>
<tr>
<td>AT4G21700</td>
<td>DUF2921 family protein</td>
<td>SAIL_172_B06</td>
</tr>
<tr>
<td>AT4G22730</td>
<td>Leucine-rich repeat protein kinase family protein</td>
<td>SALK_013143</td>
</tr>
<tr>
<td>AT4G23400</td>
<td>PCR55, Plasma membrane intrinsic protein</td>
<td>Not tested*</td>
</tr>
<tr>
<td>AT4G23850</td>
<td>LONG-CHAIN ACYL-COA SYNTHETASE 4, Fatty acid biosynthesis</td>
<td>SALK_120357</td>
</tr>
<tr>
<td>AT4G29450</td>
<td>Leucine-rich repeat protein kinase family protein</td>
<td>SAIL_337_G06</td>
</tr>
<tr>
<td>AT4G29530</td>
<td>Thiamine monophosphate phosphatase</td>
<td>SALK_101421</td>
</tr>
<tr>
<td>AT4G30190</td>
<td>PLASMA MEMBRANE PROTON ATPASE 2</td>
<td>SALK_022010</td>
</tr>
<tr>
<td>AT4G37820</td>
<td>Transmembrane protein; function unknown</td>
<td>SALK_051668</td>
</tr>
</tbody>
</table>

* Only available Salk line contains a background mutation in ped3, known to be resistant to IBA

**Table 3-2. Candidate genes mutated in Z377.**

Descriptions of candidate genes mutated in Z377 and the mutant allele tested for resistance to IBA.
3.2.c. Testing candidate genes

To test our findings in an unbiased manner, T-DNA mutants disrupting each candidate gene were acquired and screened for IBA resistance in primary root elongation (Table 3-2). Only Salk_120357 showed a comparable IBA resistant response in root elongation assays to Z377 (Figure 3-5 A). In addition to resistance in primary root elongation, Salk_120357 shows partial sensitivity to induction of lateral root formation, another phenotype consistent with Z377 (Figure 3-5 B). Salk_120357 is inserted in the 5’ UTR of LACS4 (Figure 3-6 A). Because several T-DNA alleles of lacs4 were recently named (Zhao et al. 2019), this previously uncharacterized Salk line is now named \textit{lacs4-7}.
Figure 3-5. *Salk* _120357_ and Z377 have comparable IBA resistance profiles.

(A) Primary root elongation of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by two-tailed t-test from Z377 (±SE, n ≥ 14, *p<0.001).

(B) Lateral root density of 8-day-old seedlings was quantified by dividing the number of lateral roots by the primary root length. Statistical significant determined by two-way ANOVA with *post hoc* Tukey HSD (±SE, n ≥9, p <0.001).
3.3. Confirmation that *lacs4* is the causative mutation in Z377

The mutant allele of *lacs4* identified in the screen, *lacs4*-8, is a change in nucleotide 1400 from C to T, resulting in an alanine to valine substitution at amino acid residue 467 (Figure 3-6 A). Several strategies were employed to test and demonstrate that the mutation in LACS4 is causative for the IBA resistant phenotype in Z377 and the enhanced resistance seen in Z377 *ibr3*-1. F1 noncomplementation was performed, a transgenic complementation line was created, and alternate mutant alleles of *lacs4* were compared to test if the phenotypes are similar.

3.3.a. F1 noncomplementation

To confirm causality, noncomplementation testing was completed for Z377 *ibr3*-1. F1 seedings of Z377 *ibr3*-1 backcrossed to wild type were sensitive to IBA (Figure 3-6 B), indicating that the mutation is recessive. Z377 *ibr3*-1 was crossed with *lacs4*-7. F1 progeny of this cross had intermediate resistance to IBA in primary root elongation, similar to Z377 alone, supporting that the mutation in LACS4 is causative. The individual F1 plants were genotyped at the *lacs4* mutation in Z377 and confirmed that the cross was successful.

3.3.b. Transgenic complementation

Separately, I generated a complementation line of the Z377 mutant transformed with the *LACS4* coding sequence. Z377 lines segregating a 35S:*LACS4*-GFP transgene were analyzed to observe if IBA responses were recovered by the presence of a wild-type copy of *LACS4*. Primary root length of segregating lines grown on 15 µM IBA was measured and scored for rescue relative to the IBA-resistant phenotype of Z377. The
primary root IBA resistant phenotype of Z377 is successfully complemented by a LACS4 construct, as indicated by the dual peaks representing the populations of rescued and non-rescued phenotypes within this segregating line (Figure 3-6 C). The large peak with IBA sensitive plants has root lengths less than the average root length of Z377, which suggests the addition of a wild-type LACS4 copy restores wild-type to IBA in primary root elongation. The smaller peak of the segregating population shows plants with elongated roots, with growth consistent to the Z377 mutant.

3.3.c. lacs4 alternate alleles

Causation was further demonstrated by generating novel allele combinations of lacs4 and ibr3 and looking for a similar enhanced IBA resistant response compared to the Z377ibr3-1 found in the screen. For this assay, I used lacs4-7 and also expanded the analysis to include lacs4-1, a T-DNA allele found in an exon (Figure 3-6 A) that has been previously described (Jessen et al. 2011; Jessen et al. 2015; Zhao et al. 2019). lacs4-1 and lacs4-7 were crossed separately with a T-DNA line disrupting IBR3 (Zolman et al. 2007). lacs4-1 ibr3-4 and lacs4-7 ibr3-4 have enhanced resistance to IBA in primary root elongation and indeed recapitulated the phenotype seen in the original Z377 ibr3-1 line (Figure 3-6 D). Following these tests, I concluded that the mutation in LACS4 is causative for the IBA-response defects of Z377. The Z377 mutant is now named lacs4-8 (Figure 3-6 A).
Figure 3-6. Z377 is defective in lacs4.

(A) Schematic of LACS4 gene structure. The mutant line changes nucleotide 1400 from C to T, resulting in an alanine to valine substitution at residue 467. Exons are represented as black boxes while introns are lines. Location of the T-DNA insertion mutants used in this study are depicted as inverted triangles. The causative mutation in Z377 was named lacs4-8. Predicted AMP and fatty acid binding motifs (UniProt Consortium 2019) are indicated by a star and diamond, respectively.

(B) Z377 ibr3-1 was crossed to Wt and lacs4-7 in a test of noncomplementation. F1 seedlings were grown on 20 µM IBA for 7 days. Seedlings were genotyped for the point mutation in LACS4 in Z377 to confirm the cross was successful. The Wt LACS4 allele is the smaller product while the mutant lacs4 is the larger product.

(C) Z377 was transformed with a Wt copy of LACS4. T2s were grown on 15 µM IBA for 7 days. Length of primary root was measured for each individual. Data is represented as percent of the population with indicated primary root length (n ≥18 Wt and Z377, n=32 Z377 pEG103:LACS4).

(D) Primary root elongation of 7-day-old seedlings grown on 20 µM IBA. Statistical significance was determined by one way ANOVA with post hoc Tukey HSD test (±SE, n ≥11, p <0.05). Common letters indicate no significant difference.
3.4. *lacs4* double mutant responses on IBA

To evaluate if *lacs4* enhancement of *ibr3* to IBA is specific to *IBR3* or generally enhances mutants in the pathway, double mutants combining *lacs4* with other IBA-resistant mutants were generated by outcrosses. Homozygous double mutants were tested for resistance to IBA in primary root elongation. *lacs4 ibr1-1* and *lacs4 acx3* had enhanced resistance to IBA and were statistically similar to *lacs4 ibr3* (Figure 3-7). This demonstrates that *lacs4* can generally enhance defects in IBA responses and this enhancement is not dependent on *ibr3*. 
Figure 3-7. *lacs4* generally enhances IBA resistance.

Primary root length of 7-day-old seedlings grown on 20 μM IBA. Statistical significance determined by one-way ANOVA with *post hoc* Tukey HSD (±SE, n≥8, p<0.001).
3.5. Point mutation in *lacs4*-8.

The mutant allele of *lacs4* identified in the screen, *lacs4*-8, is a change in nucleotide 1400 from C to T, resulting in an alanine to valine substitution at amino acid residue 467 (Figure 3-6 A). Despite being what is traditionally defined as a weak mutation, *lacs4*-8 yields IBA resistant phenotypes as strong as each T-DNA allele (Figure 3-6 D). To learn more about this specific mutation might disrupt LACS4 function, analysis of protein structure through bioinformatics and expression analysis was performed.

3.5.a. LACS4 expression in *lacs4*-8

To determine if *lacs4*-8 disrupts function by altered expression or protein activity, expression of LACS4 was tested by qRT-PCR using gene-specific primers. LACS4 is expressed in *lacs4*-8 at wild-type levels whereas the T-DNA mutants, *lacs4*-1 and *lacs4*-7, have dramatically reduced expression (Figure 3-8), as previously noted for *lacs4*-1 (Jessen et al. 2015). LACS4 expression is statistically the same as wild type in both *lacs4*-8 and *lacs4*-8 *ibr3*-1. This result suggests the mutation in *lacs4*-8 results in translation of a nonfunctional LACS4 protein, which is as compromised in IBA metabolism as much as plants lacking LACS4 completely.

3.5.b. Bioinformatic analysis

To gain insight into why a mutation at residue 467 disrupts function, secondary structure analysis and functional prediction was performed using Phyre2 (Kelley et al. 2015). Top models are oxidoreductases with adenylation or thioester-reduction domains, acetyl CoA synthetases, and ligases with 100% confidence that these are homologous
protein sequences, although sequence identity to all models is approximately 20%. This program suggests that residue 467 is not in a functionally important domain or region of secondary structure. The top 10 models all predict that residue 467 is in a coil structure 1-2 amino acids before a β-sheet on the external face of the protein (Figure 3-9 A). Residue 467 may hold the predicted β-sheet in a precise position for necessary function.

Mutational analysis was completed using the SuSPect method (Yates et al. 2014). Substitution to any amino acid at residue 467 is predicted to have near neutral effects and is not predicted to result in a disease state. SuSPect also predicts that the upstream amino acids are not sensitive to substitutions. However, substitutions occurring in amino acids immediately downstream of residue 467, residues predicted to make up the β-sheet, are sensitive to mutations and are predicted to result in a disease state (Figure 3-9 B).

These predictions that residue 467 is not located in a defined secondary structure, active or binding site, and is not sensitive to mutations are contrary to the genetic evidence that a substitution at residue 467 abolishes LACS4 function in IBA metabolism. This unveils questions about which regions in LACS4 are important for function and if they are equally important for lipid and IBA metabolism.

3.5.c Variation of LACS4 at residue 467

To explore if there is variation in LACS4 at this residue, I investigated a database of over 1,135 natural accessions of A. thaliana fully sequenced genomes for alterations in the coding sequence. There is no variation of residue 467 and little variation throughout the entire protein despite the prediction that variation at this residue would not disrupt protein function.
The *Arabidopsis* genome contains nine LACS genes that have similar activity and possess some defining features (Shockey et al. 2002; Hisanaga et al. 2004; Soupene and Kuypers 2008). Our analysis of LACS4 protein sequence was expanded to compare LACS4 to the other LACSs in *Arabidopsis*. The LACS4 protein sequence was aligned with the sequences of all the other *Arabidopsis* LACS proteins using T-Coffee. This revealed that residue 467 is a conserved proline (Figure 3-10 A) in all LACS proteins.

I expanded the inquiry to LACS4 orthologs in diverse species and discovered that this residue is a completely conserved proline (Figure 3-10 B). It is striking that this residue recently diverged and is conserved in *A. thaliana* but the ancestral proline is present even in *A. thaliana*’s closest relatives, *A. lyrata* and *A. halleri*. When protein alignment is compared with *A. lyrata* and *A. halleri* there are only seven non-conserved residues. These residues are either not conserved across plant clades or *A. thaliana* agrees with the majority with the exception of residue 467 which is unique. This is the only residue in *A. thaliana* that does not agree with the consensus.

Despite the SuSPect prediction that substitutions at residue 467 will not disrupt function of LACS4, there is no biological evidence of amino acid degeneracy at this residue. Residue 467 in LACS4 is a completely conserved proline in other *Arabidopsis* LACS proteins, *Arabidopsis* species, and LACS4 homologs in diverse plant species. This absolute conservation of this residue suggests that it is important for LACS4 function and may be sensitive to perturbations.

### 3.5.d. Valine and proline substitutions at residue 467 *in planta*

I directly tested if a substitution in LACS4 at residue 467 to a valine or proline disrupts LACS4 function in IBA metabolism by expressing mutant LACS4 *in planta*. I
individually transformed wild type and lacs4-7 with a copy of LACS4 containing either a valine or proline at residue 467 and a wild type copy of LACS4. I used lacs4-7 as the background mutation since the T-DNA allele has little to no expression of endogenous LACS4, therefore, all LACS4 protein is derived from the inserted transgene. In a pilot assay, lacs4-7 expressing LACS4 with a valine at residue 467, LACS4 A467V, was unable to rescue IBA resistant phenotype of lacs4-7, indicating that this LACS4 variant is nonfunctional in IBA metabolism (Figure 3-11). This is the substitution present in lacs4-8 and provides additional evidence that A467V disrupts LACS4 function and is the causative mutation in Z377. lacs4-7 containing a LACS4 transgene with a proline at residue 467, LACS4 A467P, also remained resistant to IBA indicating that this substitution disrupts LACS4 activity in IBA metabolism. Wild type expressing LACS4 A467P had a wild-type response to IBA in primary root elongation. This suggests that the endogenous copy of LACS4 in wild type is sufficient for proper IBA metabolism and that overexpression of LACS4 does not cause hypersensitivity to IBA. A wild type copy of LACS4 was able to rescue lacs4-7; this result validates the construct and experimental design.

This experimental evidence that LACS4 A467P did not rescue IBA resistance of lacs4-7 is surprising since a proline is strictly conserved in all LACS4 orthologs evaluated which suggests this variant would be functional. If LACS4 A467P is truly not functional in IBA metabolism, it is possible the role of LACS4 in IBA metabolism is newly evolved and only functions in IBA metabolism in A. thaliana but not A. halleri or A. lyrata. lacs4-7 LACS4 A467P should be evaluated to confirm the sequence and expression of the transgene before making firm conclusions. Additionally, two more
independent transgenic lines should be isolated and then tested to determine if \textit{lacs4-7} LACS4 A467P consistently remains resistant to IBA. These suggested experiments should be performed before making final conclusions about the functionality of LACS4 A467P. These transgenic lines are recently obtained T$_3$s and could not be completely evaluated due to time constraints.
Figure 3-8. LACS4 expression in lacs4 mutants.

Expression of LACS4 relative to Wt in 5-day-old seedlings grown under white light on filter paper. LACS4 expression is normalized against UBQ10. Statistical significance was determined by a two-tailed t-test (±SE, n =5, *p <0.001).
Figure 3-9. Structural prediction and mutational analysis of residue 467 of LACS4.

(A) Top model from Phyre2 (Kelley et al. 2015) structural prediction of LACS4. Secondary structure predicts α-helixes, β-sheets, and coils. Feint lines indicate coils. SS confidence indicates the confidence in the prediction with red representing high confidence and blue indicating low confidence. Predicted disordered regions are represented with a ?. Disordered confidence representing high confidence with red and blue indicating low confidence.

(B) SuSPect (Yates et al. 2014) prediction matrix for residues around 467 of LACS4. A score of 50 is the recommended cut-off between neutral and disease-causing variants, with extreme scores being more confident predictions.
Figure 3-10. Residue 467 is a conserved proline except in *A. thaliana*.

T-Coffee alignment of LACS proteins of *A. thaliana* (A) and other plant species (B). The amino acid residue mutated in *lacs4-8* is indicated with a *. Black shading indicates the same amino acid. Grey shading indicates similar amino acids.
Figure 3-11. Substitutions at residue 467 do not restore LACS4 function in planta.

(A) Primary root length of 7-day-old seedlings grown on 15 µM IBA. Statistical significance determined by one way ANOVA with post hoc Tukey HSD (±SE, n≥6, p<0.001). Common letters indicate no significant difference.
(B) Primary root length of 7-day-old seedlings grown on 10 µM IBA.
3.6. LACS4 localization

All proteins identified in IBA to IAA conversion are in the peroxisome matrix or have roles in general peroxisome function (Hu et al. 2012), inspiring the hypothesis that, based on the phenotypes described above and the potential connection of LACS4 with IBA activation, LACS4 would also have an association with peroxisomes. A previous study (Jessen et al. 2015) reported that LACS4 localizes to the ER. ER localization of a component of the IBA to IAA pathway is novel, because of this, LACS4 localization was reexamined.

3.6.a. LACS4 localization in Arabidopsis

A full-length LACS4 cDNA with a C-terminal GFP fusion with a flexible linker between LACS4 and the fluorescent tag was transformed into wild type and lacs4-8 plants. This construct rescued the IBA resistant phenotype of lacs4-8, demonstrating that the construct is functional (Figure 3-6 C). LACS4-GFP could be viewed as distinct puncta in root hairs. These puncta looked comparable in size and number to peroxisomes stained with the peroxisome specific dye, BODIPY (Landrum et al. 2010), in wild-type seedlings (Figure 3-12 A).

3.6.b. LACS4 localization in Nicotiana benthamiana

To determine if the puncta seen by LACS4-GFP in Arabidopsis were peroxisomes, Nicotiana benthamiana was co-infiltrated with Agrobacterium tumefaciens containing a 35S:LACS4-CFP with a flexible linker and UBQ10:YFP-SRL, a YFP ending with a canonical PTS1. Transient expression of LACS4-CFP could be seen as discrete
puncta that colocalized with the peroxisome marker (Figure 3-12 B). This result supports our hypothesis that LACS4 can associate with peroxisomes.

3.6.c. SRG as a noncanonical PTS1

LACS4 may merely associate with the peroxisome without being internalized. Proteins internalized into the peroxisome typically contain a PTS. LACS4 does not have a canonical PTS1 or PTS2. However, the PTS1 C-terminal tripeptide (typically [S][RK][LM]), can accept degeneracy at these residues (Chowdary et al. 2012.) The LACS4 C-terminal amino acids are SRG, one amino acid different from a canonical PTS1. To test if the C-terminal tripeptide of LACS4 functions as a noncanonical PTS, a UBQ10:YFP construct was mutated to end either with SRG or SRL terminal amino acids. YFP-SRL localized as discrete puncta (Figure 3-12 C), demonstrating peroxisome localization. In contrast, YFP-SRG was seen as a diffuse fluorescent signal (Figure 3-12 C). This experiment demonstrates that if LACS4 is entering into the peroxisome, it is doing so by a different mechanism than a C-terminal tripeptide PTS or that additional upstream residues are required beyond the consensus amino acids. Alternatively, LACS4 may associate with the peroxisome without being internalized.
Figure 3-12. LACS4 localizes to puncta reminiscent of peroxisomes.

(A) Visualization of LACS4-GFP and BODIPY stained peroxisomes in 7-day-old *Arabidopsis* root hairs. Scale bar = 20 µm.
(B) Localization of transiently expressed LACS4-CFP and YFP-SRL in *Nicotiana benthamiana*. Scale bar = 10 µm.
(C) Representative confocal images of YFP-SRL and YFP-SRG of 7-day-old transgenic *Arabidopsis* in cotyledons and roots. Scale bar = 20 µm.
3.4. LACS4 directly acts on IBA

LACS4 CoA synthetase activity on fatty acids has been demonstrated (Shockey et al. 2002). The IBA resistant phenotype of lacs4 suggests that it is also involved in IBA metabolism. To determine if the mechanism of IBA resistance of lacs4 is due to CoA synthetase activity on IBA, in vitro enzyme activity assays were performed with recombinant LACS4.

3.4.a. LACS4 activity on various IBA concentrations

A CoA synthesis reaction consumes ATP to yield pyrophosphate (PPi) and AMP (Figure 3-13 A). I examined enzyme activity by measuring the production of PPi with a fluorometric assay. 1 μg of recombinant LACS4 was incubated with increasing concentrations of IBA dissolved in water for 10 minutes in vitro. LACS4 produced significantly more PPi when incubated with IBA than when incubated with no substrate. PPi production increased with increasing concentrations of IBA (Figure 3-13 B and C). Increased PPi production could also be seen when increasing amounts of LACS4 was incubated with a constant concentration of IBA. This demonstrates that LACS4 does have activity on IBA in vitro.

3.4.b. LACS4 activity on various substrates

To evaluate substrate specificity, LACS4 was tested on IAA, IAA-alanine, methyl-IAA and tryptophan, an auxin precursor. These substrates were chosen because each are endogenous, relevant to auxin metabolism, and are structurally similar to IBA. Each possess an indole ring identical to that of IBA but have side chains of varying lengths (Figure 3-13 D). Chain length is of interest because LACS4 has activity on fatty
acids ranging in length from 14-18 carbons (Shockey et al. 2002). Methyl-IAA was included because it has a side chain the same length of IBA, but does not have a terminal carboxyl group to participate in adenylation. Despite significant LACS4 activity on IBA, activity was not detected for IAA, IAA-alanine, methyl-IAA, or tryptophan (Figure 3-13 C). This demonstrates that LACS4 has specific activity on IBA, but not other structurally similar auxins or carboxylic acids. This biochemical data is consistent with lacs4 being resistant to the effects of IBA and sensitive to IAA.

I expanded the in vitro analysis of LACS4 substrates to include 2-4-DB. 2-4-DB is a synthetic auxin and not endogenous to plants. lacs4 is weakly resistant to 2-4-DB at low levels. However, LACS4 did not have activity on 2-4-DB (Figure 3-13 D), suggesting that it is not a bona fide substrate.

Evidence that LACS4 has increasing activity with increasing IBA substrate and exhibits a specificity for IBA over related auxins strongly supports that LACS4 directly acts on IBA and can charge IBA with the necessary CoA before entering the β-oxidation pathway.
Figure 3-13. **LACS4 has activity on IBA substrate in vitro.**

(A) CoA synthetase reaction on IBA depicting intermediates and byproducts.
(B) 1 µg of recombinant LACS4 was incubated with increasing concentrations of IBA dissolved in water for 10 minutes in vitro. Production of PPi was measured and represented relative to µM PPi detected without substrate. Statistical significance was determined by a two-tailed t-test (±SE, n=6, **p<0.01, ***p<0.001).
(C) 1 µg of recombinant LACS4 was incubated with 100 µM of IBA, IAA, tryptophan (Trp), methyl-IAA (me-IAA), and IAA-alanine (IAA-ala) for 10 minutes in vitro before PPi was measured. Statistical significance was determined by ANOVA with post hoc Tukey HSD test (±SE, n=6, p<0.01). Common letters indicate no significant difference.
(D) 1 µg of recombinant LACS4 was incubated with 100 µM of IBA, 2-4-DB, or no substrate for 10 minutes in vitro before PPi was measured. Statistical significance was determined by ANOVA with post hoc Tukey HSD test (±SE, n=6, p<0.05). Common letters indicate no significant difference.
(E) Chemical structures of IBA, IAA, tryptophan, methyl-IAA, IAA-alanine, and 2-4-DB.
3.5. lacs4 and lacs4 ibr3 display reduced IBA-responsive DR5 activation in root cap

IBA conversion to IAA is an important source of auxin for root development (Strader and Bartel 2011; De Rybel et al. 2012; Xuan et al. 2015) and contributes to establishing and maintaining the total auxin pool (Spiess et al. 2014). To examine if LACS4 can influence overall auxin signaling, lacs4-8 and lacs4-8 ibr3-1 were crossed to a wild-type reporter line containing the synthetic auxin-responsive reporter DR5:GUS (Ulmasov et al. 1997). Seedlings were evaluated for relative strength of the reporter in root caps, the hypothesized location of IBA conversion into IAA (De Rybel et al. 2012; Xuan et al. 2015). In media without hormone, staining in wild type, ibr3, and lacs4-8 was comparable. DR5:GUS induction was slightly reduced in lacs4-8 ibr3-1. I hypothesize this to be due to reduced metabolism of endogenous IBA into IAA in the root cap. Upon treatment with IBA, wild-type plans show increased and expanded induction of the DR5:GUS construct. However, lacs4-8 and lacs4-8 ibr3-1 were not induced to the same levels as wild type (Figure 3-14), suggestive of reduced auxin signaling (Ulmasov et al. 1997). Staining was similar in all genotypes incubated with IAA (Figure 3-14), demonstrating that the seedlings still retain the ability to sense and respond to IAA; only the IAA generated from the breakdown of IBA is compromised. The reduced GUS induction in lacs4 supports a hypothesis that LACS4 is functionally contributing to the total auxin pool available in the root cap and is doing so through the mechanism of IBA to IAA conversion.
Figure 3-14. *lacs4* and *lacs4ibr3* display reduced IBA-responsive DR5 activation.

Staining of DR5:GUS in root tips of 5-day-old seedlings. Plants were incubated in liquid PN with or without hormone gently rocking for 2 hours. Plants were stained for GUS for 3 hours at 37 °C and washed with 50%, 75%, and 95% EtOH before imaging. Groups were imaged with a 20 X objective, insert is 40 X objective.
3.6. LACS4 relationship with LACS6 and LACS7

Nine LACS genes are encoded in the *Arabidopsis* genome (Shockey et al. 2002). As several LACS proteins have overlapping roles (Jessen et al. 2011; Jessen et al. 2015; Zhao et al. 2019) I examined all *lacs* mutants for IBA resistance. Available T-DNA lines were screened for root elongation on IBA. I found *lacs6* is the only other *lacs* mutant resistant to IBA in primary root elongation (Figure 3-15). *lacs6* was further characterized since it is also resistant to IBA. *lacs7* was also characterized because LACS6 and LACS7 are closely related, both localized to the peroxisome, and have overlapping activity in the first step of fatty acid β-oxidation (Shockey et al. 2002; Fulda et al. 2004).

3.6.a. *lacs6* and *lacs7* IBA phenotypes

*lacs6* is resistant to IBA in primary root elongation and is comparable to *lacs4*. Surprisingly *lacs7* remains sensitive to IBA and is comparable to wild type on IBA in primary root elongation (Figure 3-16 A). Evaluation of lateral root initiation in response to IBA stimulation was also examined. *lacs6* had slightly reduced lateral root density when treated with IBA (Figure 3-16 B), mimicking the same responses as *lacs4*. Lateral root density of *lacs7* grown on IBA was statistically similar to wild type, supporting that *lacs7* does not display resistance to IBA in root morphology.

To further explore the relationships between LACS4, LACS6, and LACS7, double mutants of *lacs4* with *lacs6* or *lacs7* were generated. *lacs4-7 lacs6*, but not *lacs4-7 lacs7*, has enhanced resistance to IBA in primary root elongation when compared with single mutants (Figure 3-16 A). This enhancement is not seen in the lateral root phenotype; *lacs4-7 lacs6* lateral root density is not statistically different from the single
mutants (Figure 3-16 B). This evidence supports that \textit{lacs4} and \textit{lacs6} are not defective in lateral root initiation and that their IBA defective responses are predominately seen in the primary root. The finding that \textit{lacs6} is resistant to IBA and enhances the IBA resistance in primary root elongation of \textit{lacs4} suggests that LACS4 and LACS6 may work in combination in IBA to IAA conversion. These experiments also demonstrate that LACS7 does not have a role in IBA to IAA conversion.

\textbf{3.6.b. \textit{lacs4} is not sucrose dependent, even in combination with \textit{lacs6} or \textit{lacs7}}

Redundancy is common with β-oxidation enzymes (Hu et al. 2012). Single mutants may not have sucrose dependent phenotypes even when a biochemical defect is present. For example, \textit{lacs6} and \textit{lacs7} appear wild-type but growth is severely compromised in a \textit{lacs6 lacs7} double mutant (Fulda et al. 2004). Although, \textit{lacs4} did not display sucrose dependent phenotypes (Figure 3-4), it does not rule out the possibility of a role in fatty acid β-oxidation. The overlapping role of LACS4 and LACS6 in IBA responses led us to analyze if \textit{lacs4} has a sucrose dependent phenotype in higher order mutants. \textit{lacs4-7 lacs6} and \textit{lacs4-7 lacs7} were evaluated for sucrose dependence. Neither double mutant was defective in hypocotyl elongation on media lacking sucrose, indicating \textit{lacs4} does not display the sucrose dependent phenotype consistent with fatty acid β-oxidation defects (Figure 3-16 C and D). I concluded that fatty acid β-oxidation is not detectably compromised when \textit{LACS4} is mutated.

\textbf{3.6.c. \textit{lacs6} responses to other auxins}

Evidence supports that \textit{lacs4} and \textit{lacs6} are resistant to IBA. I previously showed that \textit{lacs4} responses were specific to IBA; to explore if \textit{lacs6} responses are specific to
IBA, primary root elongation was tested on IAA and found to be inhibited similarly to wild type (Figure 3-17 A). In addition, I tested these lacs mutants on the synthetic auxin 2-4-D. lacs4 and lacs6 are sensitive to 2-4-D based inhibition of primary root elongation to the same extent as wild type (Figure 3-17 B). This indicates that neither lacs4 nor lacs6 are disrupting generalized auxin responses.

lacs4 and lacs6 were also tested on 2-4-DB, a synthetic auxin hypothesized to be β-oxidized in a similar mechanism to that of IBA (Wain and Wightman 1954). I used the peroxisomal acyl-activating enzyme, aae18, as a control as it is specific for activating 2-4-DB for β-oxidation and remains sensitive to IBA (Wiszniewski et al. 2009). lacs4 is resistant to 2-4-DB at low levels but not to the same extent as aae18 (Figure 3-17 C). Interestingly, lacs6 remained sensitive to 2-4-DB (Figure 3-17 C), consistent with previously published results (Fulda et al. 2004). Resistance to 2-4-DB was not enhanced in the lacs4-7 lacs6 double mutant, supporting that lacs6 is sensitive to this auxin. The differential effect of LACS4 and LACS6 on 2-4-DB may indicate that LACS4 preferentially participates in auxin β-oxidation compared to LACS6 or that LACS4 may be capable of accepting a broader range of substrates than LACS6.

3.6.d. lacs4 and lacs6 responses to phosphate deprivation

Under low phosphate conditions, Arabidopsis will respond by increasing lateral root growth, reducing primary root elongation, and increasing root hair length (Williamson et al. 2001; Bhosale et al. 2018). The increase in root hair length is dependent on auxin (Bhosale et al. 2018). A recent study found that ibr3 ibr1 ibr10 did not elongate root hairs to the same extent as wild type under phosphate starvation conditions (Trujillo-Hernandez et al. 2020). To evaluate lacs mutant responses and the
use of IBA in stress conditions, I assayed lacs4, lacs6, lacs4 lacs6, and lacs4 ibr3 root responses in phosphate starvation conditions.

I first evaluated if the lacs mutants were able to develop more lateral roots in phosphate starvation conditions. Wild type and all mutant genotypes had a similar lateral root density on 0.1 mM KP0₄, a low phosphate condition, and 2.5 mM KP0₄, a high phosphate condition (Figure 3-18 A). It is well documented that phosphate deprivation conditions promote lateral root development. Wild type did not have increased lateral root density in low phosphate media indicating that a lower amount of phosphate should be used or that density is not the most accurate method to describe morphogenic changes to roots under phosphate starvation conditions. Other studies often measure other parameters such as lateral root length, distance between lateral roots, distance from root tip to first lateral root, and ratio of lateral root length per total root length (Williamson et al. 2001). The use of other measurements may be necessary to capture the lateral root phenotype under low phosphate conditions.

I extended my analysis of phosphate deprivation on root architecture to root hair elongation. Wild type elongated root hairs in response to low phosphate conditions as did lacs4, lacs6, ibr3, lacs4 lacs6, and lacs4-8 ibr3-1 (Figure 3-18 B). This may indicate that ibr3, lacs4, and lacs4 ibr3 do not sufficiently disrupt IBA metabolism to the same levels as ibr3 ibr1 ibr10 to reveal defects in root hair elongation in low phosphate conditions. Alternatively, I may not have detected difference in root hair elongation due to differences in media, seedling age, or location of root hairs imaged along the primary root. This could be confirmed by including ibr3 ibr1 ibr10 in future analyses.
3.6.e. Residue 497 of LACS6

Residue 467 of LACS4 is not a defined secondary structure, active or binding site, and is not predicted to be sensitive to mutations and yet an alanine to valine substitution abolishes LACS4 function in IBA metabolism. This residue is also a conserved proline in other Arabidopsis LACS proteins and LACS4 proteins of other species (Figure 3-10). The absolute conservation suggests this residue is important for protein function although it is currently unknown the exact function and importance of this residue in LACS proteins.

In an effort to understand the role of this residue and if it is important for IBA metabolism, lipid metabolism, or general protein function I exploited the fact that LACS6 has a dual role in IBA metabolism and lipid metabolism. I mutated residue 497 of LACS6, the corresponding residue to 467 of LACS4, to be either a valine or alanine. Alanine is the amino acid located at 467 of wild-type LACS4 while valine is the mutation identified in lacs4-8. This construct was transformed into lacs6 and lacs6 lacs7 in an effort to understand if this residue or protein region is important for IBA metabolism, lipid metabolism, or general protein function. I tested if the LACS6 variants were functional and could rescue the sucrose dependent phenotype of lacs6 lacs7 and the IBA resistant phenotype of lacs6.

lacs6 lacs7 harboring the LACS6 P497A and LACS6 P497V transgenes were grown on media without sucrose to test for rescue of the sucrose dependent phenotype. lacs6 lacs7 LACS6 P497A and lacs6 lacs7 LACS6 P497V were both able to rescue the sucrose dependent phenotype of lacs6 lacs7 demonstrating that substitutions to alanine or valine does not disrupt LACS6 function in fatty acid β-oxidation (Figure 3-19).
Figure 3-15. *lacs4* and *lacs6* are resistant to IBA in primary root elongation.

Primary root elongation of 7-day-old seedlings grown on PN and PN + 10 μM IBA. Statistical significance determined by two-tailed t-test compared to Wt of same treatment (±SE, n ≥9, *p<0.05, **p<0.01, *** p<0.001).
Figure 3-16. LACS4 and LACS6 have overlapping activity in IBA metabolism but not fatty acid β-oxidation.

(A) Primary root elongation of 7-day-old seedlings on 10 μM IBA. Statistical significance was determined by one way ANOVA with post hoc Tukey HSD test (±SE, n ≥12, p <0.05). Common letters indicate no significant difference.

(B) Lateral root density of 8-day-old seedlings was quantified by dividing the number of lateral roots by the primary root length. Statistical significance determined by two-way ANOVA with post hoc Tukey HSD (±SE, n ≥14, p <0.05). Common letters indicate no significant difference.

(C) Hypocotyl elongation of seedlings grown with and without sucrose for 1 day in light and 5 days in darkness. Statistical significance determined by two-tailed t-test between sucrose treatments (±SE, n ≥17, * p <0.001).

(D) Representative hypocotyls of seedlings grown for 1 day in light and 5 days in darkness without sucrose. Scale bar = 5 mm.
Figure 3.17. LACS4 and LACS6 are specific to auxins that must be β-oxidized.

(A) Primary root elongation of 7-day-old seedlings grown with and without IAA. Statistical significance determined by two-tailed t-test to Wt of the same treatment (±SE, n ≥12, aux1-7 n=8, * p<0.001).

(B) Primary root elongation of 7-day-old seedlings grown on increasing concentrations of 2-4-D (±SE, n ≥16).

(C) Primary root elongation of 7-day-old seedlings grown on increasing concentrations of 2-4-DB (±SE, n ≥10).
Figure 3-18. *lacs4* has wild-type responses to phosphate starvation.

(A) Lateral root density of 12-day-old seedlings. Statistical significance determined by one-tailed t-test (±SE, n ≥9, p<0.05).

(B) Root hair length of 8-day-old seedlings. Statistical significance determined by one-tailed t-test (±SE, n ≥11, p<0.05).
Figure 3-19. Substitutions at residue 497 do not disrupt fatty acid β-oxidation.

Dark grown hypocotyl elongation of *lacs6 lacs7* transformed with LACS6 P497A or LACS6 P497V grown in darkness without sucrose. Data for LACS6 P497A and LACS6 P497V T2S is represented as mean hypocotyl length (A) and percent of the population with indicated hypocotyl length (B).
3.7. Discussion

IBA metabolism to IAA parallels fatty acid β-oxidation; enzymes with similar activities likely will be required for the analogous steps. It is hypothesized that IBA is charged with CoA by a CoA synthetase before entering the pathway. My identification of LACS4 in an unbiased screen for IBA resistance was intriguing, as a CoA synthetase functioning on IBA had not yet been reported. Separation of the new allele from the previously-characterized ibr3 IBA-response mutant background (Zolman et al. 2000; Zolman et al. 2007) revealed that lacs4 has a notable IBA-resistance profile on its own. Mutations in LACS4 make Arabidopsis resistant to the inhibition of IBA in primary root elongation and dark grown hypocotyl elongation (Figures 3-1 A and D), consistent with previously characterized IBA-response mutants (Zolman et al. 2000; Zolman et al. 2007; Zolman et al. 2008; Strader et al. 2011)

LACS4 CoA synthetase activity is specific to IBA in vitro. LACS4 was not active against other auxin substrates and lacs4 remains sensitive to IAA in primary root elongation (Figures 3-2 and 3-13). Our data supports a model in which LACS4 catalyzes CoA addition onto IBA, the first step in IBA to IAA conversion, and is required for normal contribution of IBA-derived IAA in the root.

3.7.a. LACS4 function

LACSs in all organisms, from prokaryotes to humans, primarily activate fatty acids with CoA making LACS enzymes critical for utilization of fatty acids (Groot et al. 1976; Watkins 1997). Arabidopsis genome contains nine LACS genes with some overlapping activity, but vary in substrate specificities, localization, and expression
Teasing apart the role of LACSs has been difficult as most single mutants have no obvious lipid phenotypes. Higher order mutants have revealed specific roles of some *Arabidopsis* LACS proteins.

LACS4 has overlapping activity with LACS1, LACS2, LACS8, and LACS9 with roles in a diverse array of lipid biosynthesis. *lacs4* appears wild-type in vegetative growth, fertility, and seed oil content (Jessen et al. 2011; Jessen et al. 2015; Zhao et al. 2019), but phenotypes began to emerge upon combining *lacs4* with other mutants. LACS4 and LACS9 act in glycerolipid synthesis and work together to transfer lipids from the ER to the plastid. *lacs4 lacs9* had reduced stature, reduced seed weight, altered seed oil content, and impaired seed development (Jessen et al. 2015; Zhao et al. 2019). LACS8 also may have overlapping activity as a *lacs4 lacs8 lacs9* triple mutant is embryo lethal (Jessen et al. 2015; Zhao et al. 2019). LACS4 and LACS1 together are required for synthesis of pollen coat lipids, as *lacs1 lacs4* shows male sterility (Jessen et al. 2011). *lacs1 lacs2 lacs4* has altered seed oil content and dramatically reduced cuticle waxes (Zhao et al. 2019). These studies link LACS4 activity to vegetative growth, seed oil accumulation, and fertility, highlighting that LACS4 has a wide impact on growth and fecundity.

Our results implicating LACS4 activity in IBA metabolism expands the roles for LACS4 to span lipid and hormone metabolism. Our work reveals LACS4 also influences early seedling development, particularly root systems, through IBA to IAA conversion and contributes to the overall pool of free IAA, as indicated by reduced induction of DR5:GUS in *lacs4-8 ibr3-1* in the root cap (Figure 3-13).
Mirroring the overlapping roles of LACS family enzymes described above, our study also implicated LACS6 as another CoA synthetase that can act on IBA. LACS6 is localized in the peroxisome matrix (Fulda 2004; Jessen et al. 2015), consistent with our initial hypothesis that the acyl-CoA synthetase acting on IBA would also be localized where all other IBA metabolizing enzymes are located (Hu et al. 2012). Multiple alleles of lacs6 are resistant to IBA in primary root elongation (data not shown) similarly to lacs4 and other ibr single mutants, supporting the hypothesis that LACS6 acts directly on IBA. Alternatively, lacs6 could indirectly disrupt IBA metabolism. Disruption to LACS6 alternatively could be indirectly influencing IBA metabolism via a mechanism such as CoA limitation, slowed peroxisome metabolism due to accumulation of fatty acid intermediates, or accepting exogenous IBA as substrate because it is in excess. Such a model might suggest lacs7 also would display IBA resistance as LACS6 and LACS7 are both highly expressed in the same tissues and have overlapping activity on fatty acid substrates (Shockey et al. 2002). However, while lacs6 is resistant to IBA, lacs7 has wild-type responses to IBA in all phenotypes tested and no additional enhancement was noted in the lacs4 lacs7 double mutant in primary root elongation or lateral root initiation assays. Future work characterizing the substrate profile of LACS6 will be necessary to establish a direct or indirect role of LACS6 in IBA metabolism.

lacs4 and lacs6 single mutants have IBA resistant phenotypes (Figure 3-15 and 3-16) whereas defects in fatty acid β-oxidation, seed oil accumulation, and glycolipid synthesis are only seen in higher order mutants with other lacs mutants (Figure 3-16 and (Shockey et al. 2002; Fulda et al. 2004; Jessen et al. 2015).
Together these findings illustrate that activity of LACS4 and LACS6 on IBA is unique, although expanded studies of this enzyme family will reveal additional details of enzyme activity and pathway interactions. For instance, *lacs8*-2 shows a slight hypersensitivity to IBA (Figure 3-15); *lacs8* is of interest as other studies report overlapping activity of LACS4 and LACS8 (Jessen et al. 2015; Zhao et al. 2019). *lacs3* also has a slight hypersensitivity to IBA but has reduced primary root length without hormone treatment, potentially indicating a general growth defect instead of an IBA specific effect. Other LACS enzymes have not been thoroughly investigated yet, but future experiments will reveal their roles on fatty acids and potentially other related substrates.

3.7.b. Additional interactions may be required for full IBA responses

*lacs4* develops lateral roots in response to IBA stimulation, although not to the extent as wild type (Figures 3-1 and 3-15); this differential pattern of resistance across developmental stages is rare in IBA-response mutants. For instance, *acx3* also develops lateral roots in the presence of IBA but is resistant in primary root elongation (Eastmond et al. 2000; Adham et al. 2005). The distinct primary and lateral root IBA-responses of *acx3* is attributed to its expression pattern. ACX3 expression in roots is seen in the root tip and tips of elongated lateral roots but not lateral root primordia, even upon IBA treatment (Eastmond et al. 2000; Adham et al. 2005).

The mechanism for LACS4 differential responses remains an open question. LACS4 and LACS6 are highly expressed in root tissue compared to other LACSs (Shockey et al. 2002) and analysis of a LACS4 promoter:GUS construct demonstrated that LACS4 expression is detected in the root tip but is absent from the elongation zone.
(Zhao et al. 2019). Analysis of LACS4 expression in lateral root primordia is required to determine if the different IBA response phenotypes of lacs4 can be attributed to cell specific expression or activity.

Interestingly, lacs6 has been previously characterized and shows sensitivity to the synthetic IBA analog 2,4-DB (Fulda et al. 2004), similar to our results (Figure 3-17 C). AAE18 is an acyl-activating enzyme found in peroxisomes with predicted overlapping activity. Notably, aae18 mutants are sensitive to IBA, but resistant to 2,4-DB (Figure 3-17 C) and (Wiszniewski et al. 2009). This unique pattern of responses represents another area for future investigation, as connections and overlap between LACS4, LACS6, and AAE18 could be revealing for different tissues or conditions as suggested by their unique but overlapping phenotypes.

3.7.c. Unique aspects of lacs4-8 mutation

The lacs4 allele discovered in our screen has a weak missense mutation of an alanine to valine. Residue 467 is not located in a known substrate or cofactor binding pocket or active site (Figure 3-6). Our structural analysis and functional prediction using Phyre2 (Kelley et al. 2015) suggests this residue is not in a functionally important domain or region of secondary structure (Figure 3-9). Our genetic evidence that a substitution at residue 467 disrupts function unveils questions about which regions in LACS4 are important for function and if they are equally important for lipid and IBA metabolism.

I also completed mutational analysis using the SuSPect method (Yates et al. 2014). Substitution to any amino acid at residue 467 is predicted to have near neutral effects (Figure 3-9). However, despite this prediction, there is no amino acid degeneracy at this residue; this residue is a completely conserved alanine in all A. thaliana accessions
and a proline in all other *Arabidopsis* LACS proteins and LACS4 orthologs in other plant species, including closely related *Arabidopsis* species (Figure 3-10). Such conservation suggests an evolutionary pressure to maintain the amino acids at this site. It is striking that LACS4 of *A. thaliana* encodes a divergent alanine at this site. The PAM250 matrix (Schwarz and Dayhoff 1979) predicts that proline is most often and equally likely to be substituted for alanine or serine. Although the recent divergence of *A. thaliana* at this amino acid is surprising, a substitution for alanine may be favorable and necessary for function. This biological evidence of strong conservation increases our confidence that this residue is sensitive to perturbations and could have strong consequences when mutated despite bioinformatic predictive analysis.

Mutations to the corresponding residue in LACS6 does not disrupt LACS6 function in fatty acid β-oxidation. LACS6 P497V and LACS6 P497A were both able to rescue the sucrose dependent phenotype of *lacs6 lacs7* demonstrating that both of these LACS6 variants are functional. This experiment demonstrates that LACS6 is not perturbed by the same mutations that disrupt LACS4. Although this result is surprising, it is not unexpected as LACS6 is unique from LACS4 in that it has defined discrete functions in fatty acid β-oxidation and peroxisome localization while LACS4 localization remains unclear and functions in a diverse array of lipid metabolic processes. Although LACS4 and LACS6 are a similar class of enzymes, they only share 40% similarity in protein sequence (Madeira et al. 2019) therefore it is not surprising they will have various response to amino acid substitutions at particular residues. One hypothesis is that residue 467 in LACS4 is necessary for recognition or binding to another protein to allow LACS4 import into the peroxisome. This analogous residue in LACS6, 497, would not be
necessary for this interaction since LACS6 has a canonical PTS, therefore LACS6 would remain functional with mutations at this residue.

Our genetic evidence supports that residue 467 is important for protein function, folding, or stability, given that a point mutation disrupts plant physiology to the same extent as null alleles (Figure 3-6 D). Continued analysis of the LACS4 domain structure generally and this region specifically is required to determine if this site is required for binding to a substrate, interacting protein, or required to give the protein a precise structure.

### 3.7.d. LACS4 localization remains an open question

In our analysis of localization, LACS4-GFP could be viewed as distinct puncta in root hairs in *Arabidopsis* (Figure 3-12). These puncta looked comparable to BODIPY stained peroxisomes. The punctate localization was also seen when LACS4-CFP was transiently expressed in tobacco and colocalized with YFP-SRL labeled peroxisomes. This evidence that LACS4 associates with peroxisomes is consistent with localization of other IBA to IAA enzymatic components (Hu et al. 2012). Subcellular localization prediction tools yield disparate predictions. WoLF-PSORT (Horton et al. 2007) and Suba4 (Hooper et al. 2017) most often predict cytosol localization followed by peroxisome localization, but predict localization to all other cellular compartments.

If LACS4 is localized to the peroxisome matrix, it is unclear how import occurs as LACS4 does not possess a canonical PTS1 or PTS2. The LACS4 C-terminal amino acids SRG are not a sufficient PTS for entry into the peroxisome (Figure 3-12), although additional upstream amino acids not tested could facilitate import (Brocard and Hartig 2006; Chowdhary et al. 2012). Some peroxisomal proteins without a PTS piggyback onto
PTS containing proteins (Thoms 2015) and protein oligomers can be imported even when subunits lack a PTS (Brown and Baker 2008). This piggybacking mechanism has also been reported for protein phosphatase 2A in *Arabidopsis* (Kataya et al. 2015) and even proteins that are not part of a complex such as including Nicotinamidase I which is co-imported into the peroxisome with glycerol-3-phosphate dehydrogenase 1 in *Saccharomyces cerevisiae* (Saryi et al. 2017).

LACS4 previously has been described as ER localized in *Arabidopsis* (Jessen et al. 2015) and Brassica napus (Tan et al. 2014). However, the mammalian homolog, ACS4, localizes to the mitochondria, ER, and peroxisomes (Lewin et al. 2001; Lewin et al. 2002; Milger et al. 2006; Grevengoed et al. 2014). This finding opens the question of LACS4 localization and the possibility of dual localization. A detailed study of LACS4 throughout development and under changing conditions, as well as identification of an interacting protein that could serve as a vehicle into the peroxisome, is necessary to elucidate the localization of LACS4 and understand the implications of potential dual localization over space and time.

### 3.7.e. Potential mechanisms of LACS4 activation of IBA

Our genetic and biochemical data support that LACS4 charges IBA with CoA. Genetic evidence suggests LACS6 does this as well. I hypothesize these two enzymes work together at this step via one of three possible mechanisms (Figure 3-19).

All enzymatic components of IBA to IAA conversion discovered to date are contained within the peroxisome. Our LACS4 localization experiments demonstrate LACS4 could be localized in the peroxisome matrix as well. This localization supports the hypothesis that LACS4 and LACS6 together charge IBA with CoA inside the peroxisome.
Alternatively, LACS4 may reside outside of the peroxisome, associated with the outer membrane. LACS4 generates a cytosolic pool of IBA-CoA which is imported into the peroxisome. Active import by PXA1 is coupled with CoA hydrolysis (Nyathi et al. 2010; De Marcos Lousa et al. 2013) and in this model, IBA could be reactivated by LACS6 in the peroxisome matrix.

However, previous work also has reported LACS4 localization in the ER (Jessen et al. 2015). LACS4 may generate a pool of IBA-CoA in the ER for import into the peroxisome, which could be reactivated by LACS6.

Finally, there could be two pools of LACS4 with distinct functions. One pool may be ER localized and function primarily in fatty acid metabolism whereas a peroxisomal pool may be primarily associated with hormone metabolism. Such a model is consistent with our mutant analysis suggesting loss of LACS4 affects IBA responses, but does not affect peroxisomal lipid metabolism during early seedling development.

Each of these proposed mechanisms have a novel aspect that is intriguing. If LACS4 is peroxisomal, it is unclear how it is imported without a canonical PTS. LACS4 could enter via the piggyback mechanism, an extended signal sequence, or a noncanonical PTS. If LACS4 is solely localized to the ER, it is the first enzyme with a role in IBA metabolism not linked to the peroxisome. However, peroxisomes have a close relationship with the ER. Peroxisomes bud off the ER membrane to become independent entities, as described in a model of de novo biogenesis, or the ER may serve as a source of membranes and membrane proteins, as described in a semi-autonomous model (Mullen and Trelease 2006; Hu et al. 2012; Agrawal and Subramani 2016). IBA-CoA generated from LACS4 in the ER may be moved into the peroxisome via these
membrane fusion models rather than active import. If LACS4 is only localized to the ER, this furthers the growing evidence that implicates the ER in auxin metabolism. IBA can be formed via acetylation of IAA and is associated with the ER membrane in maize (Ludwig-Müller and Epstein 1992; Ludwig-Müller and Hilgenberg 1995). Auxin-conjugate hydrolases reside here, IAA actively is transported into the ER, and the ER may regulate the amount of auxin transported to the nucleus for signaling (Bartel and Fink 1995; Davies et al. 1999; Friml and Jones 2010; Middleton et al. 2018).

Finally, LACS4 may exist in both the ER and the peroxisome. Multiple pools of LACS4 would accommodate the pleiotropic activity of LACS4 in lipid and IBA metabolism. Localization to multiple organelles is seen in the mammalian isoform, ACS4, which is localized to the ER, peroxisome, and mitochondrial membrane (Grevengoed et al. 2014). In mammals, superoxide dismutase (SOD1) and lactate dehydrogenase (LDH) are localized primarily to the cytosol with a smaller peroxisomal pool that enter via piggybacking mechanism (Islinger et al. 2009; De Marcos Lousa et al. 2013). The import of these proteins into the peroxisome is dependent on the amount of available carrier protein, offering an explanation for the comparatively reduced amount of protein in the peroxisome, which is often masked in in vitro studies (Thoms 2015). Dual localization of ACS4 and the ability of SOD1 and LDH to enter the peroxisome without PTSs while maintaining primary localization elsewhere substantiates the possibility that LACS4 could have also dual localization in Arabidopsis and suggests a rationale for why LACS4 does not have a PTS, why peroxisomal localization has not been previously detected, and why a significant role in fatty acid β-oxidation is not observed.
3.7.f. Summary

The roles of IBA-derived IAA, and auxin storage forms more broadly, have been investigated in detail but continue to be studied to determine the activity and relative importance of each throughout plant growth, development, and responses to changing environmental conditions. Identification of LACS4 as an activator of IBA provides a more complete view of the IBA metabolic pathway, facilitating additional studies to increase our understanding of alternative auxin input pathways during plant development.

In addition, this work gives new light to the role of LACS4 within a cell and the importance of LACS4 in plant development. LACS4 stands at the crossroads of hormone and lipid metabolism, two molecular pathways required to balance growth, development, and responses to the environment throughout the lifespan of a plant.
Figure 3-20. Three possible models of LACS4 activation of IBA, which affects IBA metabolism to IAA in the peroxisome.

(1) LACS4 and LACS6 are both located inside the peroxisome where together they charge IBA with CoA. LACS4 may localize solely to the peroxisome or also localize to the ER and/or cytoplasm.

(2) LACS4 may exist in the cytoplasm and loosely associated with the peroxisome without being internalized by the organelle to generate a cytoplasmic pool of IBA-CoA. IBA-CoA is imported into the peroxisome where LACS6 can reattach the CoA onto IBA.

(3) ER localized LACS4 may generate a pool of IBA-CoA that is actively imported into the peroxisome or is contained within pre-peroxisome structures that become mature peroxisomes. CoA cleaved off during import is reattached by the peroxisomally contained LACS6.
Chapter 4: Forward Genetic Screen for Mutants with Enhanced Resistance to IBA

A forward genetic screen for mutants that are resistant to the effects of IBA revealed a diverse array of phenotypic profiles and, through much work, led to the identification of enzymatic components of IBA to IAA conversion. In addition, the screen shed light on intricacies of peroxisome function and revealed that peroxisome matrix dynamics might influence internal pathways. These previous studies unveiled the understanding of IBA metabolism we have today.

Although many components specific to IBA metabolism and peroxisome function have been identified, our understanding is incomplete. I sought to identify additional components of IBA metabolism by continuing the forward genetic screen for plants resistant to IBA. To increase our chances of finding novel components, an enhancer screen was performed.

4.1. Screen for enhanced resistance to IBA

The mutants, ibr3-1 and ibr1-1, defective in IBA β-oxidation enzymes, were remutagenized with EMS to generate a new population of mutants that could unveil additional genetic interactions. ibr3-1 and ibr1-1 are resistant to both the inhibition of IBA on root elongation and the stimulatory effect of IBA on lateral root initiation (Zolman et al. 2007; Zolman et al. 2008), common IBA resistant traits. ibr3 was chosen as the background because it displays weaker IBA resistance than other ibr mutants (Zolman et al. 2008), facilitating the potential to capture a range of enhancement phenotypes, from subtle to considerable.
IBA does effect hypocotyl elongation but has been a trait less screened for because it is requires more experimental steps and a higher concentration IBA. Resistance to IBA in hypocotyl elongation has exposed new discoveries. *ech2* was only discovered through forward genetics in a screen for hypocotyl resistance to IBA (Strader et al. 2011). To increase discovery of new genes, I screened for mutants that have enhanced resistance to IBA inhibition of dark grown hypocotyl elongation. I hypothesized this screen could facilitate the identification of factors involved in the function or regulation of IBR3 or IBR1, the conversion of IBA to IAA, and/or general peroxisome function in *Arabidopsis*.

Approximately 15,000 mutagenized M$_2$ seedlings were screened of each mutant background. Pooled M$_2$ seeds were grown on 60 µM IBA in darkness and screened for any seedling with a hypocotyl longer than the average. Mutants with long hypocotyls were rescued to soil to continue to the next generation. 71 mutants were pulled from the *ibr3*-1 enhancer screen and 56 were pulled from the *ibr1*-1 screen. Mutants recovered from the enhanced hypocotyl resistance to IBA screen hypocotyls were named hypocotyl resistance to IBA (HRI) followed by a number. An example of the enhanced hypocotyl resistance to IBA seen in the HRI mutants is depicted in Figure 4-1.

### 4.2. Confirmation of enhancer phenotypes

Mutants rescued from the initial screen were retested on IBA in the M$_3$ generation to confirm the IBA resistant phenotype. Lines that did not continue to display IBA resistance in dark grown hypocotyls were eliminated. After rescreening, six mutants in the *ibr3*-1 background and five in *ibr1*-1 background were retained for further characterization. These mutants were further characterized by evaluating their responses...
to IBA in root elongation. Some mutants from the HRI *ibr1-1* screen displayed varying resistance to IBA in hypocotyls vs roots (Figure 4-2). For example, HRI #11 *ibr1-1* has enhanced resistance to IBA in roots but not hypocotyls, while HRI#18 *ibr1-1* and HRI#41 *ibr1-1* have enhanced resistance to IBA in hypocotyls but not roots. Varied IBA responses in tissues have not been seen before. Later generations following a backcross to wild type should be further examined to confirm if these phenotype are tractable and true. Mutants selected for further characterization based on tractability and phenotypes are listed in Table 4-1.

### 4.3. Separation of new mutation from background

Mutants were backcrossed to wild type to segregate non-causative mutations and to attempt to separate the new mutant from the parent mutant background. F₂ seedlings were grown on 60 µM IBA and screened for individuals with enhanced resistance to IBA and individuals with intermediate resistance to IBA comparable to *ibr3* or *ibr1*. F₂ progeny were characterized at a genotypic and phenotypic level. Mutants displaying the enhanced IBA resistance are theoretically the double mutant selected from the original screen. Individuals with intermediate IBA resistant phenotype could be *ibr3* or *ibr1* single mutants or the unknown mutant alone. These plants with intermediate resistance to IBA were genotyped at *IBR3* and *IBR1*. A subset genotyped wild-type at the *IBR3* or *IBR1* locus. These lines are hypothesized to represent the effects of the new mutation alone. The separated single mutants and parent double mutants were evaluated for IBA resistance to roots in the F₃ generation (Figure 4-3). Some HRI mutations were successfully separated from the *ibr3-1* or *ibr1-1* background and are resistant to IBA alone, such as HRI #11, HRI #18, HRI #27 (Figure 4-3). Some of these single mutants...
were not resistant to IBA which indicates that particular individual might have been incorrectly genotyped and is likely wild type, explaining the wild-type like phenotype. The phenotype and genotype of the single mutants should be scrutinized carefully in the next generation to determine if the mutation truly was separated from the mutant background. Details of lines that were tractable and the most promising are outlined in Table 4-1.

HRI mutants were evaluated for lateral root responses to IBA stimulation. HRI#55 single mutant was uniquely able to develop lateral roots in response to IBA (Figure 4-4). Dissimilar responses to IBA in primary root and lateral root development is uncommon. Most IBA resistant seedlings are insensitive to IBA in all relevant developmental responses, but this different response to IBA in primary root and lateral roots is seen in the acx mutants (Adham et al. 2005). I hypothesize that HRI#55 may be defective in one of the ACX genes.
Figure 4-1. Example enhancer mutants pulled from screen.

Representative picture of 6-day-old dark grown hypocotyls on 60 µM IBA.
Putative HRI ibr1-1 M₃ mutants were evaluated for resistance to IBA in hypocotyl and root elongation. Hypocotyl elongation of seedlings grown on 60 μM IBA for 1 day in light and 5 days in darkness was measured for HRR ibr1-1 mutant lines (A), (C), and (E). Root length of 7-day-old seedlings was measured for HRI ibr1-1 mutant lines grown on 30 μM IBA (B), (D), and (F).
Figure 4-3. Separation of HRI mutation from the ibr3-1 background.

(A) Root length of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by a two-tailed t-test to Wt (±SE, n≥7, * p<0.01, ** p<0.001).

(B) Root length of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by a two-tailed t-test to Wt (±SE, n≥10, * p<0.01, ** p<0.001).
Figure 4-4. Lateral root density of HRI mutants.

Lateral root density of 8-day-old seedlings was quantified by dividing the number of lateral roots by the primary root length. Statistical significance was determined by one-way ANOVA with post hoc Tukey HSD. Common letters indicate no significant difference (±SE, n ≥7, p <0.01).
Table 4-1. Mutants retained from enhancer screen.

Mutants have enhanced resistance to IBA in hypocotyl elongation. Table includes what pool of M2 seedlings the mutants were screened from, genotypes the mutant was crossed to, if the new mutation could be separated from the mutant parent background, if the mutant was sequenced, and any additional notes.
4.4. Identification of mutants

The strategy to identify the causative mutation in the enhancer mutants is a combined approach of mapping and whole genome sequencing. Rough mapping was used to narrow down the location of the causative mutation to a chromosome or region of a chromosome. Identifying a general location of the gene of interest will complement the sequencing data and make its analysis more manageable by only evaluating mutations in a particular genomic region.

4.4.a. Identification of HRI #55

*HRI*#55 ibr3-1 was backcrossed to wild type. Individuals were identified that were resistant to IBA but genotyped wild-type at the *IBR3* locus in the F$_2$ generation. These individuals were evaluated for several IBA resistant phenotypes in the F$_3$ generation. These single mutants were resistant to IBA in primary root elongation, but sensitive to IBA in lateral root initiation. This differential pattern of resistance across developmental stages is rare in IBA-response mutants, but has been seen in *acx* mutants (Eastmond et al. 2000; Adham et al. 2005). *acx*3 IBA response have been characterized and is known to develop lateral roots in the presence of IBA but is resistant in primary root elongation (Eastmond et al. 2000; Adham et al. 2005). The distinct primary and lateral root IBA-responses of *acx*3 is attributed to its expression pattern. *ACX3* expression in roots is seen in the root tip and tips of elongated lateral roots but not lateral root primordia, even upon IBA treatment (Eastmond et al. 2000; Adham et al. 2005). The similarity of *HRI*#55 and *acx*3 in this phenotype profile inspired the hypothesis that the causative mutation in *HRI*#55 might be in an *ACX*. 
To quickly determine if ACX3 is mutated in *HRI*#55, ACX3 of the *HRI*#55 mutant was sequenced. There was a C to T mutation consistent with EMS mutagenesis at position 2204 that caused a nonsense mutation. The glutamine at residue 382 was changed to a stop codon resulting in a truncated protein.

Based on the similar phenotypes of *HRI*#55 and *acx3*, and that *acx3* was identified as the causative mutation in another enhancer mutant, Z353 *ibr3-1* (described in Chapter 5), I conclude that *HRI*#55 is defective in ACX3 and is now named *acx3-8* (Figure 5-1). *acx3-8 ibr3-1* is further investigated in Chapter 5.
4.4.b. Mapping

Enhancer mutants were outcrossed to the Wassilewskija (WS) ecotype of Arabidopsis thaliana to generate a genetic mapping population. Outcrossed F2 seedlings were grown on 60 µM IBA and screened for mutants that retained the enhanced resistance to IBA in hypocotyls. Screening for the double mutant was necessary because I have more confidence screening the enhanced IBA resistant phenotype and because an intermediate resistance to IBA could be caused by the ibr3 or ibr1 mutations or the new mutation, with potential to lead me astray. DNA was extracted from at least 20 F2 mutants and genotyped at markers that can distinguish Col vs WS DNA. Recombination frequency was calculated for each marker. Low recombination frequency is evidence of more Col DNA in that particular region of the chromosome indicative of a selective pressure to maintain genes in that region. This reduced recombination frequency is expected in regions of the chromosome near the mutations that cause the resistance to IBA.

Mutants, HRI #70 ibr3-1 and HRI#11 ibr1-1, were outcrossed to WS. Recombination frequency was calculated for F2 mutants with enhanced resistance to IBA at several genomic markers. HRI#70 ibr3-1 had a recombination frequency of 2.9 % at marker GAPC located on the top of chromosome 3, which is expected as this is the location of IBR3. This gives us confidence in the mapping population because it demonstrates low recombination frequency at the expected region. Six genomic markers were tested and did not have a reduced recombination frequency to further investigation of these regions. Markers tested and recombination frequency are listed in Table 4-2.
HRI#11 ibr1-1 F₂ mutants outcrossed to WS were also evaluated at several genomic markers. Two markers, F19P19 and MRN17, had the lowest recombination frequencies that were less than 40 %. F19P19 is located on the top of chromosome 1 with the lowest recombination frequency of 33 % and MRN17 is located at the top of chromosome 5 with a recombination frequency of 40 %. Markers tested and recombination frequency for each are listed in Table 4-2. These two regions of the genome became the focus of additional scrutiny in the whole genome sequencing data.
### Table 4-2. Recombination frequency of markers in *HRI#70ibr3-1* and *HRI#11ibr1-1*.
Location of the genomic markers tested and recombination frequency at each marker is included.
4.4.c. Whole genome sequencing

Whole genome sequencing was used as a method to identify new mutations introduced by the EMS mutagenesis in an effort to identify the causative mutation for the enhanced resistance to IBA. \textit{HRI}#11 \textit{ibr}1-1, \textit{HRI}#41 \textit{ibr}1-1, and \textit{HRI}#70 \textit{ibr}3-1 were selected for whole genome sequencing. \textit{ibr}1-1, \textit{ibr}3-1, and wild type were sequenced in parallel to serve as references and to identify background mutations.

4.4.c.i. Sequencing populations

\textit{HRI}#11 \textit{ibr}1-1 and \textit{HRI}#41 \textit{ibr}1-1 was backcrossed to wild type to segregate as many non-causative mutations as possible. The resulting F\textsubscript{2} population was screened for enhanced resistance to IBA in dark-grown hypocotyls. Tissue from 24 \textit{HRI}#11 \textit{ibr}1-1 seedlings and 31 \textit{HRI}#41 \textit{ibr}1-1 seedlings that were confidently scored for the enhanced phenotype was used to extract DNA for whole genome sequencing. DNA from a segregating population would also allow us to employ a mapping by sequencing strategy to narrow down the location of the causative mutation (Hartwig et al. 2012).

\textit{HRI}#70 \textit{ibr}3-1 was also backcrossed to wild-type and the F\textsubscript{2} population was screened for individuals with enhanced resistance to IBA. These individuals were carried out to the next generation. The F\textsubscript{3} plants were rescreened for the enhanced IBA resistant phenotype to confirm they were homozygous and had the desired phenotype. Each of these F\textsubscript{3} lines segregate unlinked mutations at random and uniquely from each other. This generates diversity in the sequencing population to help eliminate non-causative homozygous mutations. 15 mg of seeds from eight independent \textit{HRI}#70 \textit{ibr}3-1 F\textsubscript{3} were grown for 10 days. These seedlings were pooled together and DNA was extracted from the bulked seedlings.
4.4.c.ii. Sequencing results

**HRI #70 ibr3-1**

Sequencing data from HRI#70 ibr3-1 revealed an EMS mutation in ACX3 that introduced a premature stop codon. This mutation is the exact same mutation found in HRI#55 ibr3-1. It is unlikely that the exact same point mutation was independently generated by EMS in both HRI#55 ibr3-1 and HRI#70 ibr3-1. It is more likely that HRI#55 ibr3-1 was mislabeled as HRI#70 ibr3-1 in an earlier generation or was accidentally pulled from the same pool of seeds as HRI#55 ibr3-1 even though it is documented that they were screened from different pools of seeds (Table 4-1). Genotyping primers could be designed to detect mutations that are present in HRI#70 ibr3-1 in unrelated genes chosen at random. If the same mutations are present in HRI#55 ibr3-1 but not ibr1-1 or wild type, I would conclude that HRI#70 ibr3-1 and HRI#55 ibr3-1 are indeed, the same line that has experienced a mislabeling event.

**HRI#11 ibr1-1**

Candidate mutations in HRI#11 ibr1-1 were first narrowed by identifying homozygous SNPs consistent with EMS mutagenesis in protein coding regions. 17 homozygous EMS mutations were identified. These candidates were further narrowed by eliminating mutations that were common between ibr1-1 which left only two candidates: AT2G08986, a hypothetical protein, and AT3G49640, a tRNA-dihydouridine synthase (Table 4-3). AT2G08986 is a hypothetical protein with no known function and very low expression that appears to be confined to the shoot apical meristem (SAM) according to the eFP browser (Klepikova et al. 2016). The expression profile of AT2G0898 makes this
gene unlikely to be a true candidate. AT2G08986 contains five EMS mutations in HRI#11 ibr1-1 but contains 35 mutations in ibr1-1. The high accumulation of changes in this gene suggest there is no pressure to maintain genetic integrity at this loci and AT2G08986 is unlikely to be a functional gene. I could not make a clear hypothesis linking the functions of AT2G08986 or AT3G49640 to IBA metabolism or peroxisomes, therefore, the list of candidate mutations was expanded.

Sequencing data for HRI#11 ibr1-1 was generated from a pooled F2 population that may accidentally include DNA from non-enhancer plants. Inclusion of any number of non-enhancers in the sequencing population may result in incorrect scoring of homozygous vs heterozygous mutations. Out of an abundance of caution, all EMS SNPs are considered candidates for the causative mutation that results in enhanced resistance to IBA. There are 244 EMS SNPs in 154 unique genes in HRI#11 ibr1-1. This list of candidate genes was compared to a list of known peroxisomal genes and auxin related genes. HRI#11 ibr1-1 had mutations in two of the known candidate genes: PXA1 and MPK5 (Table 4-3).

HRI#11 ibr1-1 has an EMS generated SNP in PXA1 that results in an alanine to a valine substitution. This mutation in PXA1 is scored as being heterozygous. Despite being scored as heterozygous, this mutation could be causative and should be investigated further. I hypothesize that this mutation is not the causative mutation in HRI#11 ibr1-1 because the alanine is substituted for a similar amino acid and this exact mutation is also seen in the sequencing data from ibr1-1. Mutations common between enhancer mutants and either wild type or the ibr1-1 background are unlikely to be causative.
The substitution in \textit{MPK5} in HRI\#11 \textit{ibr1-1} is a glutamic acid to a lysine. This is a significant amino acid change and could be causative. However, this mutation is unlikely to be the causative mutation in HRI\#11 \textit{ibr1-1} because this mutation in also detected in \textit{ibr1-1}.

The exact substitutions seen in \textit{PXA1} and \textit{MPK5} are also detected in the sequencing data from \textit{ibr1-1} which is cause to eliminate them as candidates. Both \textit{PXA1} and \textit{MPK5} are located on chromosome 4, near \textit{IBR1}. Because of the proximity of \textit{PXA1}, \textit{MPK5}, and \textit{IBR1} to each other in the genome, these genes are linked making it difficult to for the mutations in \textit{PXA1} and \textit{MPK5} to segregate away from \textit{IBR1}. Therefore, it is expected that the mutations in \textit{PXA1} and \textit{MPK5} will remain present as background mutations in all HRI mutants from the \textit{ibr1-1} screen. I conclude that the mutations in \textit{PXA1} and \textit{MPK5} are unlikely to be the cause of the enhanced resistance to IBA seen in HRI\#11 \textit{ibr1-1}.

There are no other obvious candidate mutations in HRI\#11 \textit{ibr1-1}. Another strategy to narrow down the location of the causative mutation is to evaluate the distribution of mutations across the genome. The causative mutation likely lies in a region of the genome with an accumulation of nearby mutations due to gene linkage (Hartwig et al. 2012). Chromosome one of HRI\#11 \textit{ibr1-1} contains the most EMS mutations with 77 changes. Chromosome four contains the second highest number of mutations with 54, but is expected to maintain a high number of mutations due to linkage effects surrounding \textit{IBR1}. Chromosome one and five are the largest chromosomes in the \textit{Arabidopsis} genome, are comparable in size, and would be expected to contain a larger number of mutations than the other chromosomes due to their size alone. Chromosome
five contains only 35 EMS mutations. The distribution of mutations across the chromosomes of HRI#11 ibr1-1 suggest the causative mutation might be located on chromosome one. This is consistent with the rough mapping data that the lowest recombination frequency was detected on the top of chromosome one (Table 4-2). However, each of the EMS mutations on chromosome one is also detected in ibr1-1 and are likely all background mutations.

**HRI#41 ibr1-1**

Candidate mutations in HRI#41 ibr1-1 were first narrowed by identifying homozygous SNPs consistent with EMS mutagenesis in protein coding regions. Only 12 homozygous EMS mutations were identified. These candidates were further narrowed by eliminating mutations that were common between ibr1-1 which left only one candidate, AT2G08986. AT2G08986 is the same hypothetical protein found in HRI#11 ibr1-1 and highly mutated in ibr1-1. Although it is possible AT2G08986 is the causative mutation in HRI#41 ibr1-1, other candidates should be considered.

Sequencing data for HRI#41 ibr1-1 was also generated from a pooled F2 population that may include individuals not resistant to IBA. Therefore, all EMS SNPs can be considered as possible causative mutations. There are over 1,400 other EMS SNPs in HRI#41 ibr1-1 to be considered as candidates. Genes with mutations in HRI#41 ibr1-1 were compared to a list of known peroxisomal genes and auxin related genes. HRI#41 ibr1-1 contains mutations in seven known peroxisome genes and eight auxin related genes (Table 4-3). The mutations in PXA1 and MPK5 seen in ibr1-1 and HRI#11 ibr1-1 are among this list, but are eliminated due to these mutations being present in the ibr1-1
background. From the list of known candidate genes, the mutations in AAE12 and GH3.15 are of particular interest.

AAE12 is of interest because AAE18 is hypothesized to be the CoA synthetase required for 2-4-DB β-oxidation (Wiszniewski et al. 2009). *aae18* is resistant to 2-4-DB but sensitive to IBA. It is possible that other acyl activating enzymes behave in the opposite manner, functioning on IBA but not 2-4-DB or act on both hormones (Wiszniewski et al. 2009).

The mutation in GH3.15 is the most intriguing candidate because this is the acyl acid amido synthetase that conjugates glutamine to IBA (Sherp et al. 2018). One mutant allele of *gh3.15* was slightly hypersensitive to IBA (Sherp et al. 2018), consistent with the hypothesis that these plants are not be able to inactivate IBA and, therefore, have a stronger response to the hormone. Although, a hypersensitive response to IBA is expected in a knockout *gh3.15*, it does not eliminate the possibility that a mutation in GH3.15 will perturb IBA homeostasis in unexpected ways leading to resistance to IBA. It is also possible that the *gh3.15* mutation in HRI#41 *ibr1-1* results in a gain of function and would be hypothesized to cause resistance to IBA. However, the serine to asparagine substitution is not considered a strong mutation.

Hypotheses can be made for the mutations in AAE12 and GH3.15 of HRI#41 *ibr1-1* causing the enhanced resistance to IBA. Analyzing these candidate mutations to determine if they are homozygous in individual HRI#41 *ibr1-1* plants confidently scored with the enhanced resistance to IBA phenotype is a starting point to determine if they are indeed causative.
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<td>E/K</td>
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**Candidate mutations in HRI#11ibr1-1**

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**Candidate mutations in HRI#41ibr1-1**

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<td>Aldolase</td>
<td>R/H</td>
</tr>
</tbody>
</table>

**Table 4-3. Candidate mutations in HRI#11ibr1-1 and HRI#41ibr1-1**

List of candidate genes containing non-synonymous EMS generate SNPs in protein coding sequences of HRI#11 ibr1-1 and HRI#41 ibr1-1 including genes with known functions to peroxisomes or auxin responses.
4.4.c.iii. Conclusion

Even with employing multiple strategies, identifying causative mutations in the enhancer mutants was challenging. Although mapping is an effective method, rough mapping did not provide sufficiently detailed information to narrow down the location of the new mutation. More markers would need to be tested to find regions with reduced recombination frequency. Although informative, mapping will cost time to gather this data.

Sequencing data is an excellent method to give a landscape of mutations in the genome, although identifying the single SNP leading to the phenotype can be arduous. Sequencing a segregating F2 population has the advantage of segregating non-linked mutations in a short amount of time. However, after these experiments, I conclude the risks outweigh the benefits. One or two plants phenotypically scored incorrectly could cause the causative mutation to be scored as heterozygous in the sequencing results, increasing the chances for the gene to not be identified as a candidate. Waiting until plants reach the F3 generation allows the plants to be retested for the phenotype, increasing confidence in the population. Pooling independently derived F3 gives enough genetic diversity to segregate non-causative mutations. Complete confidence in the sequencing population is worth the time until the F3 generation.

4.5. Discussion

The forward genetic screen for mutants with enhanced resistance to IBA in hypocotyl elongation was successful in finding several mutants with this phenotype from
both the ibr3-1 and ibr1-1 background. This suggests that there are many genes that contribute to IBA metabolism and many ways to disrupt this process.

Some of the new mutations were successfully separated from the ibr3-1 or ibr1-1 background and are resistant to IBA on their own. HRI#11, HRI#18, HRI#27, and HRI#55 were separated from ibr3-1 and are resistant to IBA in primary root elongation. Mutations to these individual genes can independently influence IBA metabolism do not rely on the presence of ibr3 to disrupt IBA metabolism. HRI#55 had the unique ability to initiate lateral roots in the presence of IBA. This phenotype is reminiscent of acx3 (Adham et al. 2005). acx3 was already identified as the causative mutation in a screen for roots with enhanced resistance to IBA which made ACX3 a likely candidate. Sequencing of ACX3 in HRI#55 revealed a premature stop codon, confirming that this is the causative mutation.

Recognizing the unique phenotype of acx3 in HRI#55 and discovering the mutation in a single sequencing reaction was a stroke of luck. At the time, the acxs were the only genes known to be resistant to IBA in primary root elongation but not lateral root initiation. It was not long after identifying HRI#55 that lacs4 was confirmed as the causative mutation in Z377, which also develops lateral roots in the presence of IBA. Even though the varying IBA responses in primary versus lateral roots is rare, the number of mutant genes in this category is expanding.

Identifying the causative mutations in the HRI enhancer mutants was challenging. I found a large number of EMS SNPs in protein coding regions in the HRI mutants. In part, this is because these mutants are expected to have a high number of background mutations as ibr3-1 and ibr1-1 were generated by EMS in the original screen for IBA.
resistance (Zolman et al. 2000) and were mutagenized with EMS a second time for this screen. The high number of expected EMS mutations emphasizes the importance of the population used for sequencing.

It is important to segregate as many non-causative EMS SNPs as possible while maintaining confidence the causative mutation is homozygous to reduce the number of candidate mutations to sort through. Sequencing DNA from pooled F<sub>2</sub> individuals scored for the enhancer phenotype was not stringent enough to ensure the causative mutation is homozygous. In the future I recommend sequencing pooled DNA from independently isolated F<sub>3</sub> lines from a backcross to generate genetic diversity in unlinked regions of the genome while being able to test the phenotype of the population to ensure it is homozygous.

Although I have sequencing data that lists all mutations in HRI#11 ibr1-1 and HRI#41 ibr1-1, I have not successfully identified the causative mutation that enhances ibr1-1. There are no clear candidate genes in HRI#11 ibr1-1. HRI#41 ibr1-1 has mutations in two intriguing candidate genes, AAE12 and GH3.15. Alternate mutant alleles of aae12 and gh3.15 can be obtained and tested for IBA resistance to determine if these genes influence IBA metabolism. If either are resistant to IBA, they can be crossed to HRI#41 ibr1-1 in a test of non-complementation. Alternatively, genotyping primers can be designed to detect the SNPs in AAE12 and GH3.15. HRI#41 ibr1-1 individuals can be genotyped at these loci to determine if aae12 or gh3.15 are homozygous in all individuals or segregate. Experimental evidence that the mutation is homozygous in all individuals tested could indicate that the mutation might be causative and warrants further investigation.
When there are no candidate mutations to test, as in HRI#11 ibr1-1, more extensive mapping could be done with a segregating mapping population. Mapping would narrow down the location of the causative mutation. When a general genomic location is determined, the sequencing data could be reevaluated. All mutations in that localized region could be considered candidates. Thus far, only guanine (G) to adenine (A) and cytosine (C) to thymine (T) transitions have been evaluated as these are the most frequent mutations induced by EMS treatment. EMS is known to induce A to G and T to C transitions, insertions, and deletions at a lower frequency than guanine alkylation (Sega 1984). Analysis of candidate genes can be expanded to include these types of mutations in protein coding regions. The mutants could also be sequenced again using a pooled DNA from F3 populations.
Chapter 5: ACX3

Z353 ibr3-1 is a mutant pulled from a previous screen for enhanced resistance to IBA in primary root inhibition and was identified as being defective in acx3 by Dr. Zolman. The Z353 allele is now named acx3-7 (Figure 5-1). Independently, I identified acx3-8 as the causative mutation in HRI#55 ibr3-1 from the screen I conducted for enhanced resistance to IBA in hypocotyl elongation. Identifying acx3 twice in screens for enhanced resistance to IBA highlights its importance and warrants further investigation.

ACX3 functions in the oxidation step of fatty acid β-oxidation (Froman et al. 2000; Eastmond et al. 2000), but acx3 is also resistant to IBA (Adham et al. 2005). acx3 was discovered in an unbiased screen for IBA resistance (Adham et al. 2005) and was already known to enhance IBA resistance when combined with ibr3 through reverse genetics (Zolman et al. 2007). Although it has been known for some time that acx3 is resistant to IBA, the role of ACX3 in IBA metabolism remains unclear.

5.1. acx3 ibr3 phenotypes

ACX3 is one of six peroxisomal acyl-CoA oxidases that participates in fatty acid β-oxidation (Eastmond et al. 2000; Graham and Eastmond 2002; Graham 2008). In vitro assays demonstrate that ACX3 has a preference for medium chain length fatty acids (Eastmond et al. 2000; Froman et al. 2000). The role of ACX3 in fatty acid β-oxidation is well understood. However, acx3 has been identified in a previous forward genetic screen for IBA resistance and the acx family is known to have varying degrees of resistance to IBA (Adham et al. 2005). The role of ACXs in IBA metabolism is unclear. In efforts to understand ACX3, I began by characterizing the new acx3 alleles discovered in the screens for enhanced resistance to IBA.
5.1.a. IBA resistant phenotypes

*acx3 ibr3* was already discovered to have enhanced resistance to IBA in primary root elongation (Zolman et al. 2007). I wanted to characterize the newly discovered *acx3* alleles to determine if they have similar IBA resistance profiles. A new allele combination of *acx3-6 ibr3-9* was generated as the mutations in both genes are T-DNA insertions. Both *acx3-6* and *ibr3-9* have been backcrossed to wild type and will give us a clear phenotype free from any potentially interfering EMS mutations in the background. *acx3-7 ibr3-1, acx3-8 ibr3-1,* and *acx3-6 ibr3-9* have similar IBA resistant enhancement and *acx3-7* and *acx3-6* have comparable resistance in primary root elongation, demonstrating that the mutations in *acx3* similarly disrupt ACX3 function (Figure 5-2 A and B).

The enhanced resistance to IBA extends to hypocotyl elongation. *acx3-8 ibr3-1* was pulled from a screen for hypocotyl resistance to IBA and retains a significantly longer hypocotyl when grown on very high concentrations of IBA (Figure 5-2 C).

*acx3* still maintains the ability to initiate lateral roots (Figure 5-3 and (Adham et al. 2005). The new *acx3* alleles were examined for their ability to initiate lateral root when grown on IBA. All *acx3* single mutants were able to develop some lateral roots but at a reduced density to that of wild type (Figure 5-3). *acx3 ibr3* were not able to develop lateral roots when stimulated with IBA and were statistically comparable to wild type grown without hormone. Therefore, this demonstrates that the *ibr3-1* phenotype of defective lateral root development is epistatic to that of *acx3*. 
5.1.b. Sucrose dependence

acx3 enhances phenotypes of ibr3, therefore, I wanted to test if the reverse is also true: that ibr3 may enhance phenotypes of acx3 in fatty acid β-oxidation. An acx3 single mutant is not sucrose dependent (Adham et al. 2005). A phenotypic defect in fatty acid β-oxidation is not seen in acx3 due to functional redundancy or compensation by the other ACXs since higher order acx1 acx3 acx4 mutants are sucrose dependent (Rafeiza et al. 2012). A potential role of IBR3 in fatty acid β-oxidation may only be revealed in higher order mutants. acx3-8 ibr3-1 and acx3-6 ibr3-9 have comparable hypocotyl growth on media with and without sucrose, indicating that fatty acid β-oxidation is not compromised (Figure 5-4). These results supports that ibr3 does not disrupt fatty acid β-oxidation at a phenotypic level. There is currently no evidence that IBR3 influences fatty acid β-oxidation directly or indirectly.

5.1.c. Assaying CoA oxidase activity

Phenotypic analysis of acx3 ibr3 suggests that defects in IBA metabolism are exacerbated in the double mutant, but fatty acid β-oxidation is not, as acx3 ibr3 has enhanced resistant to IBA but is not sucrose dependent. Absence of a sucrose dependent phenotype does not eliminate the possibility that a defect in fatty acid β-oxidation is present due to the overlapping activities of the ACX family. Therefore, I tested plant extracts for acyl-CoA oxidase activity against fatty acids of different chain lengths to determine if there are defects at an enzymatic level.

Extracts from 4-day-old wild type, ibr3, acx3, and acx3 ibr3 seedlings were assayed for their ability to oxidize n-hexanoyl-CoA (C6:0), lauroyl-CoA (C12:0), and oleoyl-CoA (C18:1). acx3 ibr3 had consistently reduced oxidase activity when incubated
with C6:0. Surprisingly, acx3 single mutants appeared more impaired than any acx3 ibr3 double mutant (Figure 5-5 A and B). This experiment was only conducted once; more replicates would have to be conducted to confirm these results.

acx3 extracts incubated with C12:0 had reduced activity compared to wild type as expected, since ACX3 has the most activity against fatty acids of 8-14 carbons in length (Eastmond et al. 2000; Froman et al. 2000). ibr3 had slightly reduced activity relative to wild type. This result should be confirmed with additional replicates and other alleles. acx3 ibr3 extracts incubated with C12:0 had no detectable activity, was dramatically reduced when compared to wild type or acx3 single mutants, and was consistent across acx3 alleles, as acx3-7 ibr3-1 and acx3-8 ibr3-1 have little to no activity on C12:0 (Figure 5-5 C and D). I have confidence that extracts from acx3-7 ibr3-1 and acx3-8 ibr3-1 are not compromised, as they are the same extracts used in Figure 5-5 A which have some activity against C6:0, demonstrating that enzymes in the extracts do have activity.

Extracts were also tested on C18:1. acx3-6 ibr3-9 also has reduced activity when compared to wild type and acx3-6 on this substrate (Figure 5-5 E and F). ibr3-1 had little to no activity on C18:1, which is surprising. Possible reasons for these results are that extracts from ibr3-1 were not handled properly and enzymes are now nonfunctional or ibr3-1 is very important for oxidizing oleoyl-CoA. Another possibility is that the extract has a very low concentration of enzymes, therefore, biasing the comparison to the other extracts.

Assays of CoA oxidase activity of seedling extracts were pilot experiments and the data is not well supported. Despite this concern, acx3 ibr3 has consistently reduced CoA oxidase activity against all fatty acid substrates and suggests that there may be
enhanced defects in fatty acid β-oxidation at an enzymatic level. Much more work would need to be done to confirm this.
**Figure 5-1. Gene schematic depicting mutations in acx3 used in this study.**

*acx3*-6 is a T-DNA located in the fourth intron of ACX3. *acx3*-7 is a G to A point mutation at nucleotide 336 that changes a glutamic acid to lysine at residue 137. *acx3*-8 is a C to T mutation at position 2204 that caused a nonsense mutation at residue 382.
Figure 5-2. *acx3* enhances *ibr3-1*.

(A) Primary root elongation of 7-day-old seedlings grown on increasing concentrations of IBA (±SE, n ≥7).
(B) Primary root elongation of 7-day-old seedlings grown on increasing concentrations of IBA (±SE, n ≥9).
(C) Hypocotyl length of seedlings grown on media with and without sucrose for 1 day in light and 5 days in darkness (±SE, n ≥6).
Figure 5-3. *acx3* still develops lateral roots.

Lateral root density of 8-day-old seedlings was quantified by dividing the number of lateral roots by the primary root length. Statistical significance was determined by two-way ANOVA with *post hoc* Tukey HSD (±SE, n ≥8, p < 0.001). Common letters indicate no significant difference.
Figure 5-4. *acx3 ibr3* is not sucrose dependent.

Hypocotyl length of 6-day-old seedlings grown for 1 day in light and 5 days in darkness. Statistical significance determined by two-tailed t-test (± SE, n ≥11, p<0.05).
Figure 5-5. Acyl-CoA oxidase activity on fatty acid substrates.

µM H₂O₂ produced over time from 100 µg total protein from extracts from 4-day-old seedlings with 0.125 mM n-hexanoyl-CoA (A) and lauroyl-CoA (C). Average rate of µM H₂O₂ produced per minute from extracts incubated with n-hexanoyl-CoA (B) and lauroyl-CoA (D). Statistical significance determined by two-tailed t-test from Wt (±SE, n=1, * p<0.05, ** p<0.001).

(E) µM H₂O₂ produced over time from 200 µL total protein extracts from 4-day-old seedlings with and 0.125 mM oleoyl-CoA.

(D) Average rate of µM H₂O₂ produced per minute from (F). Statistical significance determined by two-tailed t-test from Wt (±SE, n=1, ** p<0.001).
5.2. Possible roles of ACX3 in IBA metabolism.

It is already known that *acx* mutants are resistant to IBA and some higher order mutant combinations become increasingly more resistant to IBA (Adham et al. 2005; Rafeiza et al. 2012). ACX3 and IBR3 are currently placed catalyzing the same oxidation step in their respective pathways and *ibr3* and *acx3* have comparable IBA resistant phenotypes (Figure 5-2), highlighting the similarity of these two enzymes. Two hypotheses are proposed to explain the role of ACX3 in IBA metabolism: ACX3 and IBR3 are catalytically redundant and both function in IBA metabolism or *acx3* indirectly disrupts IBA metabolism (Figure 5-6).

The first hypothesis is that ACX3 functions redundantly with IBR3 and directly catalyzes IBA-CoA into 2-trans-indole-3-butyryl-CoA. *ibr3* displays weaker IBA resistance than other *ibr* mutants (Zolman et al. 2008). This could explained by weak compensation by ACX3 and explains why *acx3* enhances *ibr3*. It is possible that ACX3 and IBR3 are similar enzymes capable of accepting the same substrates. The alternative explanation is that there is reduced access to substrates and necessary cofactors in the peroxisomal environment of the *ibr3 acx3* double mutant. Both ACX3 and IBR3 are predicted to have similar enzymatic functions acting on substrates charged with CoA and require shared cofactors. Blocks in fatty acid β-oxidation may cause intermediates to accumulate enhancing physical constraints and impair access to substrates and cofactors, as well as reduced availability of CoA, a limiting component in the pathway.
5.2.a. acx3 enhances other ibrs

If ACX3 is functioning in IBA to IAA redundantly with IBR3, the enhanced IBA resistant phenotype should be limited to that combination of double mutants. Higher order mutants combining acx3 with different ibrs and ibr3 with other fatty acid β-oxidation mutants were generated to test this hypothesis. IBR1 and IBR10 function downstream of IBR3 in IBA to IAA conversion. ibr1 acx3-6 and ibr10 acx3-6 double mutants were generated to determine if acx3 enhancement is specific to ibr3. ibr3-1 acx3-6, ibr1-1 acx3-6, and ibr10-1 acx3-6 all similarly elongated primary roots when grown on IBA (Figure 5-7 A). acx3 enhances IBA resistance when combined with other known ibr genes, indicating that enhancement is nonspecific.
*acx3* may disrupt IBA metabolism indirectly via a mechanism of cofactor limitations or accumulation of pathway intermediates. Alternatively, the ACX proteins may have overlapping activity with IBR3 in IBA metabolism. Disruption to *ACX3* and IBR3 halt IBA β-oxidation with only minor disruptions to fatty acid β-oxidation due to compensation by the functional ACXs.

**Figure 5-6. Models of ACX3 action in IBA metabolism.**

*acx3* indirectly disrupts IBA metabolism

IBA β-oxidation

\[
\text{IBA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA}
\]

Fatty acid β-oxidation

\[
\text{Fatty acid} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA}
\]

**acx3** directly disrupts IBA metabolism

IBA β-oxidation

\[
\text{IBA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA} \rightarrow \text{IBA CoA}
\]

Fatty acid β-oxidation

\[
\text{Fatty acid} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA} \rightarrow \text{Fatty acid CoA}
\]
5.2.b. *ibr3* enhances other *acxs*

ACXs all function as oxidases in fatty acid β-oxidation, but have distinct preferences for fatty acids of certain chain lengths (Eastmond et al. 2000; Froman et al. 2000). Their discrete substrate profiles may extend to IBA, as *acxs* have varying resistances to IBA. *acx1* and *acx3* display the strongest defects to IBA responses followed by *acx4* and have enhanced IBA resistance when these mutations are combined, while *acx2* and *acx5* have weak resistance to IBA (Adham et al. 2005; Rafeiza et al. 2012). *ibr3* was crossed to each *acx* to determine if *ibr3* similarly enhances the *acxs*. *ibr3-1 acx1* and *ibr3-1 acx3* had clear enhanced resistance to IBA compared to *ibr3* alone (Figure 5-7 B). *ibr3-1 acx4* appears to have slightly enhanced resistance to IBA when compared with *ibr3-1*. *ibr3-1 acx5* was statistically the same as *ibr3-1*, demonstrating that *acx5* has a reduced role in IBA metabolism than the other *acxs* (Figure 5-7 B). The different degrees of IBA resistance seen in *ibr3 acx* double mutants is likely due to the influence of the individual *acxs* with equal contribution by *ibr3*. This experiment agrees with previously published results (Adham et al. 2005) that the ACXs may have different contributions to IBA β-oxidation, but does not provide clear evidence if ACXs directly function in IBA metabolism. If ACXs do function as oxidases in IBA β-oxidation, ACX1, ACX3, and ACX4 are the only family members that function in both IBA and fatty acid β-oxidation. ACX1 and ACX3 have overlapping activity on medium length fatty acids (Eastmond et al. 2000) and is consistent with the hypothesis that they would also have overlapping activity on IBA.
5.2.c. *ibr3 acxs are not sucrose dependent*

*ibr3* in combination with other *acx* have varying degrees of enhanced resistance to IBA. To determine if *ibr3* enhances defects in fatty acid β-oxidation from the *acx* mutations, *ibr3 acx* double mutants were assayed for their dependence on sucrose. *ibr3-1 acx3, ibr3-1 acx4, and ibr3-1 acx5* elongated root similarly when grown on media without sucrose and media containing sucrose, suggesting these mutants are not dependent on sucrose (Figure 5-7 C). *ibr3-1 acx1* had reduced root length on media containing sucrose which is likely due to poor germination or an unrelated factor. This experiment should be repeated and assayed for dark grown hypocotyl length, as is traditionally assayed. *ibr3-4 acx1-2* and *ibr3-9 acx3-6* were assayed for sucrose dependent growth in dark grown hypocotyls and found to elongate hypocotyls similarly in media with and without exogenous sucrose (Figure 5-7 D). These experiments support that *ibr3* does not enhance defects in fatty acid β-oxidation when combined with any *acx* mutation. There continues to be no evidence that IBR3 functions in fatty acid β-oxidation.

5.2.d. **Endogenous complementation**

Transgenic complementation of *ibr3* with *ACX3* or *acx3* with *IBR3* respectively is the most direct test to determine if *ACX3* and *IBR3* have similar activity *in planta*. Previous attempts to complement *ibr3* with *ACX3* have failed due to silencing of the *ACX3* gene, likely due to its extremely high expression under the 35S promoter. Expressing *ACX3* at endogenous levels may be more appropriate. *ACX3* and *IBR3* have different expression profiles in root tissue (Eastmond et al. 2000; Adham et al. 2005). To
account for different expression patterns, I designed promoter swapping constructs that put ACX3 and IBR3 under control of the others promoter. I generated constructs that put ACX3 cDNA under control of its own endogenous promoter ACX3 promoter:ACX3 cDNA (Ap:Ac) or the IBR3 promoter, IBR3 promoter:ACX3 cDNA (Ip:Ac). Parallel constructs were made with IBR3, with IBR3 under the control of the IBR3 promoter, IBR3 promoter:IBR3 cDNA (Ip:Ic) and ACX3 promoter, ACX3 promoter:IBR3 cDNA (Ap:Ic). ibr3-1 was transformed separately with the Ip:Ac and Ap:Ac constructs to determine if ACX3 can genetically complement ibr3. The Ip:Ic was transformed into ibr3 as a control to confirm that the construct is functional. acx3-6 was transformed with Ap:Ic and Ap:Ac.

IBA resistance of ibr3-1 was not rescued by transformation with Ip:Ac, indicating that ACX3 cannot function in place of IBR3 in IBA metabolism. ibr3-1 Ip:Ac were either comparable to ibr3-1 in IBA resistance or had slightly enhanced resistance. The ibr3-1 Ip:Ac lines with enhanced resistance to IBA may be caused by silencing of ACX3. Ip:Ac was also unable to rescue the IBA resistant phenotype of acx3. Three independently transformed lines in the acx3-6 background displayed the same IBA resistance as acx3 (Figure 5-8) demonstrating that the transformed ACX3 cDNA construct may not be functional.

One acx3-6 Ap:Ic line was tested for IBA resistance. This transgenic line had roots that were IBA resistant similarly to acx3 and did not rescue the phenotype.

Wild type transformed with Ap:Ac became resistant to IBA and ibr3-1 transformed with Ap:Ac displayed enhanced resistance to IBA. This indicates that the Ap:Ac construct silenced the expression ACX3, acting like a knockout instead of a low
overexpression. This construct was ineffective at inducing a slight overexpression of ACX3. Overexpression of ACX3 under a 35S promoter is functional in the T2 generation and can rescue the phenotype of acx3, but results in silencing of ACX3 in the T3 generation. To determine if the Ap:Ac is functional in the T2 generation, acx3 AP:Ac was evaluated for rescue of the IBA resistance phenotype in the T2 generation. Ap:Ac transformed into acx3-6 did not appear to rescue IBA resistance in T2s, seeding doubt that the construct is functional at all.
Figure 5-7. acxs enhance IBA resistance but ibrs do not enhance sucrose dependence.

(A) Primary root length of 7-day-old seedlings. Statistical significance determined by a one-way ANOVA with a post hoc pairwise t-test (±SE, n ≥8, p <0.05).

(B) Primary root length of 7-day-old seedlings. Statistical significance determined by a one-way ANOVA with post hoc Tukey HSD (±SE, n ≥11, Wt n=3, p <0.05).

(C) Root length of roots grown on media with 0.5% sucrose and without. Statistical significance determined by one-tailed t-test (±SE, n ≥5, p <0.05).

(D) Hypocotyl length of seedlings grown for 1 day in light and 5 days in darkness. Statistical significance determined by one-tailed t-test (±SE, n ≥8, p <0.05).
Figure 5-8. Ip:Ac does not rescue IBA phenotypes of acx3 or ibr3.

(A) Root length of 7-day-old seedlings grown on 12.5 μM IBA. Statistical significance determined by one way ANOVA with post hoc Tukey HSD (± SE, n ≥7 acx3-6 n=5, p<0.05).

(B) Root length of 7-day-old seedlings grown on 12.5 μM IBA. Statistical significance determined by one way ANOVA with post hoc Tukey HSD (± SE, n ≥9, p<0.05).
Figure 5-9. Ap:Ic does not rescue IBA phenotype of acx3.

Root length of 7-day-old seedlings grown on 12.5 μM IBA. Statistical significance determined by one way ANOVA with post hoc Tukey HSD (± SE, n ≥9 acx3-6 n=5, p<0.05).
Figure 5-10. Ap:Ac does not rescue acx3 and silences endogenous ACX3.

(A) acx3-6 was transformed with Ap:Ac. T2S were grown on 15 µM IBA for 7 days. Length of primary root was measured for each individual. Data is represented as percent of the population with indicated primary root length (acx3-6 Ap:Ac n ≥26, Wt and acx3-6, n ≥14).

(B) Root length of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by one way ANOVA with post hoc t-test (±SE, n >9, p≤ 0.01).

(C) Root length of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by one way ANOVA with post hoc Tukey HSD (±SE, n >9, p≤ 0.01).
5.3. Alleviating peroxisome CoA limitations

The alternative hypothesis to explain why \textit{acx3} is resistant to IBA is that it does so through an indirect mechanism. One proposed mechanism is that available CoA in the peroxisome becomes limiting. IBA metabolism, fatty acid β-oxidation, and JA metabolism all are initiated with the addition of CoA onto the substrate. Blocks in these pathways may lead to accumulation of acyl-CoA ester intermediates that never complete β-oxidation to release the substrate and acetyl-CoA. Blocks such as this may prevent CoA recycling. In a limited space, such as the peroxisome matrix, such limitations may be more likely to perturb other pathways in the same environment. To test this hypothesis, I attempted to increase peroxisomal CoA.

5.3.a. PXN

Peroxisomes must import all necessary enzymes, substrates, and cofactors required for internal metabolic pathways. The peroxisomal NAD carrier (PXN) is capable of transporting NAD$^+$, NADH, AMP, ADP, and CoA (Agrimi et al. 2012; Bernhardt et al. 2012; van Roermund et al. 2016). Improper levels of these cofactors are enough to disrupt peroxisomal metabolic processes, as \textit{pxn} was found to be defective in fatty acid β-oxidation and is resistant to 2-4-DB at low concentrations (Bernhardt et al. 2012).

I tested \textit{pxn} for resistance to IBA to determine if defects in cofactor import are enough to disrupt IBA metabolism. \textit{pxn} remains sensitive to IBA in primary root elongation (Figure 5-11 A). This indicates that defects in this carrier are not sufficient to disrupt IBA β-oxidation or has little effect.
I attempted to increase free peroxisomal CoA by overexpressing PXN. If a CoA limitation is the mechanism by which acx3 is IBA resistant, increased CoA levels will theoretically rescue defects in IBA metabolism. PXN was overexpressed with the 35S promoter in wild type and acx3. The ATP import protein, PNC1, was overexpressed as a control for perturbations to small molecule peroxisome import.

35S:PXN was unable to rescue the IBA defective response in primary root elongation of acx3-6 (Figure 5-11 B). acx3-6 35S:PXN was comparable to acx3-6 or has a slightly enhanced resistance to IBA. Further study indicating that overexpression of PXN does not increase IBA metabolism and potentially disrupts IBA. More work would need to be done to confirm the phenotypic results and determine if NAD+, NADH, AMP, ADP, or CoA levels in the peroxisome increased with PXN overexpression.

Overexpression of PNC1 relieved the IBA defective responses of acx3-6 and the primary root of acx3-6 35S:PNC1 was statistically similar to wild type when grown on IBA (Figure 5-11 C). This experiment does not conclusively demonstrate that overexpression of PNC1 rescues acx3. More independent transgenic lines would need to be tested to see if the rescue is a trend. It is also important to scrutinize wild type overexpressing PNC1 to test if wild type also becomes more sensitive to IBA.

5.3.b. ACH2

An additional approach to increase free peroxisomal CoA is to overexpress an acyl-CoA thioesterase, which will hydrolyze CoA charged fatty acids into their individual components, increasing CoA and substrate. ACH2 was chosen because it is insensitive to feedback inhibition by free CoA and under normal conditions is expressed in mature
tissues (Tilton et al. 2004), suggesting that this thioesterase will have a lesser impact on fatty acid β-oxidation.

It is important that ACH2 does not function in IBA metabolism. The intention is to overexpress ACH2 to increase levels of CoA by hydrolysis from fatty acids instead of hydrolysis from IBA-CoA intermediates. ach2 was evaluated for resistance to IBA to determine if this thioesterase has any activity on IBA. ach2 remained sensitive to IBA and is comparable to wild type, suggesting it does not have a role in IBA metabolism (Figure 5-12). I can then conclude that ACH2 overexpression should not affect IBA metabolism by hydrolyzing IBA intermediates. I therefore expect effects of ACH2 overexpression on IBA metabolism to be due to an indirect mechanism.

ACH2 was overexpressed in wild type, ibr3-1, acx3-6, and ibr3-9acx3-6 backgrounds. Overexpression of ACH2 did not clearly rescue IBA phenotypes of acx3-6 or ibr3-9 acx3-6 (Figure 5-13 C). Primary roots of acx3-6 35S:ACH2 grown on IBA were comparable to acx3-6 or were slightly enhanced. One line of ibr3-9 acx3-6 35S:ACH2 grown on IBA was comparable to ibr3-9 acx3-6, while the other two lines had reduced primary root length on media with and without IBA (Figure 5-13 D). Reduced growth on media without IBA suggests that overexpression of ACH2 causes a general growth defect. However, 35S:ACH2 in wild type has comparable primary root elongation to wild type with and without IBA (Figure 5-13 A), indicating that the reduced growth of ibr3-9 acx3-6 35S:ACH2 may be due to seed quality or another variable. These seeds would need to be retested in the next generation to determine if the reduced growth is a true phenotype. One line of ibr3-1 35S:ACH2 had primary root growth comparable to wild type on IBA (Figure 5-13 B). This experiment would need to be repeated along with
genotyping to confirm the ibr3-1 mutant background and verify the presence of the transgene.

Based on these results, it does not appear that overexpression of ACH2 rescues the IBA resistance seen in acx3.

5.3.c. PXA1

Enzymes and substrates destined for the peroxisome are imported into the internal matrix through PXA1 (Zolman et al. 2001a; Hayashi et al. 2002). Active import of CoA charged substrates by PXA1 is coupled with CoA hydrolysis (Nyathi et al. 2010; De Marcos Lousa et al. 2013). Transgenic overexpression of PXA1 is another potential mechanism to increase peroxisomal CoA. PXA1 was overexpressed in wild type, ibr3, acx3-6, and ibr3-9 acx3-6 backgrounds and IBA responses were examined.

Overexpression of PXA1 in wild type appears to generally reduce primary root length, as average root length is significantly different than wild type without the transgene (Figure 5-14 A). This result was seen in three independent transgenic lines. However, on IBA, primary root length of two independent transgenic lines is comparable to wild type. A third PXA1 overexpression line is slightly resistant to IBA. This experiment would need to be repeated to see if overexpression of PXA1 does result in IBA defects.

Overexpression of PXA1 in ibr3-1 in two independent lines is statistically the same as ibr3-1 grown with and without IBA (Figure 5-14 B). This experiment suggests that overexpression of PXA1 does not generally affect plant growth and that the growth defects seen in the wild type background are artifacts.
Figure 5-11. Effects of overexpression of PXN on IBA metabolism.

(A) Root length of 7-day-old seedlings grown on specified concentrations of IBA. Statistical significance was determined by a one-tailed t-test from Wt on same treatment (±SE, n ≥7, p<0.05).

(B) Root length of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by one-way ANOVA with post hoc Tukey HSD (±SE, n ≥8, p<0.05).

(C) Root length of 7-day-old seedlings grown on 10 µM IBA. Statistical significance determined by one-way ANOVA with post hoc Tukey HSD (±SE, n ≥9, p<0.01).
Figure 5-12. *ach2* is not resistant to IBA.

Root length of 7-day-old seedlings grown indicated concentrations of IBA. Statistical significance determined by one-tailed t-test from *Wt* on same treatment (±SE, n ≥7, p<0.05).
Figure 5-13. Overexpression of ACH2 does not rescue IBA resistance.

(A) Primary root elongation of 7-day-old seedlings. Statistical significance determined by one tailed t-test from Wt on the same treatment (±SE, n ≥7, *p <0.05).

(B) Primary root elongation of 7-day-old seedlings. Statistical significance determined by one tailed t-test from ibr3-1 on the same treatment (±SE, n ≥6, ***p <0.001).

(C) Primary root elongation of 7-day-old seedlings. Statistical significance determined by one tailed t-test from acx3-6 on the same treatment (±SE, n ≥7, *p <0.05).

(D) Primary root elongation of 7-day-old seedlings. Statistical significance determined by one tailed t-test from ibr3-9 acx3-6 on the same treatment (±SE, n ≥7, *p <0.05, **p<0.01, ***p<0.001).
Figure 5-14. Overexpression of *PXA1* in wild type and *ibr3-1*.

(A) Primary root length of 7-day-old seedlings. Statistical significance determined by one tailed t-test from *Wt* on corresponding treatment (±SE, n ≥5, *p*<0.05, **p**<0.01).

(B) Primary root length of 7-day-old seedlings. Statistical significance determined by one tailed t-test from *ibr3-1* on corresponding treatment (±SE, n ≥8, *ibr3-1 35S:PXA1 3* n=4, ***p**<0.001).


5.4. Discussion

ACX3 is one of six acyl-CoA oxidases that function in peroxisomal fatty acid β-oxidation (Eastmond et al. 2000; Graham and Eastmond 2002; Graham 2008). In addition to the role of ACX3 in fatty acid β-oxidation, ACX3 may function as an oxidase in IBA metabolism as acx3 is resistant to the effects of IBA in primary root elongation.

5.4.a. ACX3 identification in unbiased screens

acx3 was identified in a unbiased screen for plants with resistance to IBA 15 years ago (Adham et al. 2005) and was already known to enhance IBA resistance when combined with ibr3 (Zolman et al. 2007). In recent years, two screens for mutants with enhanced resistance to IBA were conducted. Only three enhancer mutants have been identified from those screens and two of those mutants were new alleles of acx3. Identifying acx3-7 and acx3-8 in unbiased screens for enhanced resistance to IBA highlights the importance of acx3 in IBA metabolism and ability to enhance ibr3. acx3 ibr3 is strongly resistant to IBA at high concentrations (Figure 5-2) in both roots and hypocotyls. Several alleles of ibr3 and ibr1 have been identified in screens for IBA resistance (Zolman et al. 2007; Zolman et al. 2008) while only one allele of ibr10 (Zolman et al. 2008) and ech2 (Strader et al. 2011) have been identified, while others have never been identified through forward genetics. It is hypothesized that IBA-CoA oxidation is the rate limiting step in IBA metabolism and is crucial for proper metabolism. Disruptions to this step hinder IBA metabolism, leading to identifying IBR3 many times. If ACX3 functions as an IBA-CoA oxidase in IBA metabolism in parallel
with IBR3, it is conceivable that ACX3 would also be identified frequently in forward genetic screens.

However, ACX3 functioning as an oxidase that directly catalyzes the oxidation of IBA-CoA is only one hypothesis for its IBA resistant phenotype. An alternative hypothesis is that ACX3 may be indirectly disrupting IBA metabolism through another mechanism such as CoA limitations (Adham et al. 2005; Rafeiza et al. 2012). If the mechanism of \textit{acx3} enhancement of \textit{ibr3} is due to disruptions to internal peroxisome dynamics, then peroxisome metabolism is very sensitive to perturbations; a major point to consider when conducting future research on peroxisomes.

The hypothesis that \textit{acx3} indirectly disrupts IBA metabolism is supported by the fact that the members of the ACX enzyme family exhibit specificity towards substrates of particular chain lengths (Eastmond et al. 2000; Froman et al. 2000; Adham et al. 2005). Generalist activity on IBA is inconsistent with demonstrated specificity on fatty acid substrates.

Determining which hypothesis for the role of ACX3 in IBA metabolism is correct has been challenging, as genetics were the main tool at the time these experiments were conducted. Results from many experiments are ambiguous or could support either hypothesis.

5.4.b. \textit{acx ibr} phenotypes

\textit{acx3 ibr3} is strongly resistant to IBA in both primary root and hypocotyl elongation (Figure 5-2). The new point mutants \textit{acx3}-7 and \textit{acx3}-8 have comparable phenotypes alone and when in combination with \textit{ibr3} (Figure 5-2 and 5-3). \textit{acx3}-7 and \textit{acx3}-8 have the unique ability to develop some lateral roots on IBA but do not when
combined with ibr3, suggesting that ibr3 is epistatic to acx3 in lateral root development. All acx3 alleles have comparable phenotypes (Figures 5-1 through 5-4), giving us confidence that the mutations in acx3 are indeed causative and that acx3 influences IBA metabolism. However, the details of ACX3 of secondary protein structures necessary for function, such as substrate binding sites or domains specific for influencing IBA metabolism, have yet to be revealed.

acx3 ibr3 is not sucrose dependent (Figure 5-3). Double mutants combining ibr3 with other acxs are also not sucrose dependent (Figure 5-7 C and D), further supporting the findings and provides evidence that IBR3 does not function in fatty acid β-oxidation. This suggests that although acx3 enhances the IBA resistance of ibr3, ibr3 does not enhance acx3 in fatty acid β-oxidation.

Even though a sucrose dependence phenotype is not seen in acx3 ibr3, the defect in fatty acid β-oxidation may actually be enhanced, since extracts from acx3 ibr3 are consistently slower to metabolize fatty acids in vitro (Figure 5-5). This inability to metabolize fatty acids is most pronounced on C12:0 (Figure 5-5 C and D). C12:0 is the substrate ACX3 is documented to have the most activity against (Eastmond et al. 2000; Froman et al. 2000). Assaying the CoA oxidase activity of plant extracts requires more work to optimize the assay and replicates to confirm these results. Possible reduced activity of ibr3 on fatty acids should be confirmed by including additional alleles in the assay. There is currently no evidence that IBR3 has enzymatic activity on fatty acids or a role in fatty acid β-oxidation.

Phenotypes of enhanced resistance to IBA are not specific to the acx3 ibr3 combination. acx3 in combination with ibr1 and ibr10 also enhance IBA resistance
compared to $acx3$ (Figure 5-7). $acx3$ can enhance IBA resistance when combined with any step of the IBA metabolic process. Similar to these results, $ibr3$ enhances the IBA resistance of other $acxs$ (Figure 5-7). $ibr3$ in combination with the $acxs$ have variably enhanced IBA resistance, consistent with the general IBA resistance of the single $acxs$. $acx1$ and $acx3$ have the strongest resistance to IBA as single mutants (Adham et al. 2005; Rafeiza et al. 2012) and $ibr3$ $acx1$ and $ibr3$ $acx3$ display the most enhanced resistance to IBA. These results are consistent with the variable influences of the ACXs on IBA metabolism.

5.4.c. in planta manipulation of ACX3

Attempts to manipulate expression of $ACX3$ in planta has been challenging. Transgenic lines designed to overexpress $ACX3$ result in silencing of $ACX3$ expression altogether. A stable transgenic line overexpressing $ACX3$ has not been successfully generated in the past or in this work. Even a construct designed to express $ACX3$ under its endogenous promoter (Ap:Ac) silences $ACX3$, as wild type Ap:Ac becomes resistant to IBA and $ibr3$-1 Ap:Ac has enhanced resistance to IBA (Figure 5-10). This construct may not function as intended, as Ap:Ac does not appear to rescue the IBA resistant phenotype of $acx3$. This suggests that the construct does not function or the experiment measuring for a rescued phenotype was not representative and should be repeated.

A promoter swapping experiment was conducted in planta to determine if $ACX3$ can function in place of IBR3 and vice versa. $ACX3$ expressed from the IBR3 promoter was unable to rescue the IBA resistant phenotypes of $acx3$ and $ibr3$ (Figure 5-8). $ibr3$ Ip:Ac is still resistant to IBA and suggests that $ACX3$ can not function in place of IBR3 while $acx3$ Ip:Ac suggests that expression patterns between IBR3 and ACX3 might be too
different for ACX3 to rescue ibr3. These results suggest that ACX3 is differentially expressed than IBR3 and does not have overlapping function with IBR3 in IBA metabolism. However, results from the Ap:Ac construct raise doubt that the Ap:IC and Ip:Ac constructs are functional. Expression analysis and protein quantification would need to be done to determine if these constructs are functional and if the data from these transgenics is supported.

The inability to generate genetic tools to manipulate ACX3 in planta severely limits our ability to make conclusions about ACX3 in the physiological responses of plants to IBA. Even after generating many transgenic lines designed to alter ACX3 expression, I can still only conclude that acx3 has an IBA resistant phenotype, silences when overexpressed, and enhances the IBA phenotype of ibr3. Transgenic manipulation of ACX3 has been unsuccessful at determining the mechanism of ACX3 influence on IBA metabolism. Future work may rely on in vitro assays to determine the mechanism of ACX3 influence on IBA metabolism.

5.4.d. Increasing the pool of peroxisomal-free CoA

To test the hypothesis that acx3 indirectly influences IBA metabolism by reducing availability of free CoA, transgenic lines were generated to attempt to increase levels of free CoA in the peroxisome. I overexpressed PXN, the hypothesized transporter that brings CoA into the peroxisome; ACH2, an acyl-CoA thioesterase that cleaves CoA off other molecules; and PXA1, the primary peroxisome transporter that hydrolyses CoA off substrates upon import.

Overexpression of PXN did not rescue the IBA resistant phenotype of acx3 and pxn was not resistant to IBA at low concentrations (Figure 5-11). After these transgenic
lines were generated, another study reported that *PXN* has low affinity for CoA and is unlikely to import CoA under physiologically relevant conditions (van Roermund et al. 2016). Overexpression of *PXN* is unable to increase levels of peroxisomal CoA as intended. *PXN* does import NAD and AMP (Agrimi et al. 2012; De Marcos Lousa et al. 2013; van Roermund et al. 2016). If the overexpression line increased import of these molecules into the peroxisome matrix, it can be concluded that they are not limiting factors in IBA metabolism.

Overexpression of *ACH2* was intended to increase levels of CoA by cleaving off CoA from other substrates. 35S:*ACH2* did not rescue IBA resistance of *acx3* or *ibr3 acx3* and appears to enhance the resistance of *acx3* (Figure 5-13). Without quantifying peroxisomal CoA and other substrates in the peroxisome, these results are ambiguous and can be interpreted in multiple ways.

If 35S:*ACH2* did increase CoA levels, I could conclude that CoA limitation is not the mechanism of *acx3* perturbation of IBA metabolism. Overexpression of *ACH2* appears to enhance the IBA resistance of *acx3*. If peroxisomal total protein, substrate, and cofactor levels are increased, it suggests that peroxisome internal pathways require a delicate balance of internal components to function efficiently. It would be interesting to test if *ACH2* overexpression lines increase sucrose dependence in any genetic background.

Overexpression of *PXA1* did not appear to influence IBA metabolism in wild type or *ibr3* (Figure 5-14). Homozygous *acx3* 35S:*PXA1* lines were not completed, therefore, I cannot say for certain if overexpression of *PXA1* would rescue *acx3*. Evidence that wild type did not become hypersensitive to IBA suggests that effects of 35S:*PXA1* on IBA
metabolism is minimal and may not manipulate the pathway as much as may be required to test the indirect role of ACX3.

A genetic approach to manipulate internal peroxisome components has not yielded clear results to support or refute either hypothesis of the role of ACX3 in IBA metabolism.

5.4.e. Conclusions

acx3 ibr3 is a mutant with strong enhanced resistance to IBA affecting all known physiological responses of IBA. Despite the genetic evidence that ACX3 influences IBA metabolism, the mechanism remains elusive. Two hypotheses have been developed, one citing a direct role of ACX3 in metabolizing IBA and the other an indirect role. The experiments described here were designed to determine which hypothesis is supported but have not provided enough evidence to conclusively support or refute either.

Expression of ACX3 in place of IBR3 in planta was intended to determine if ACX3 can function in IBA under endogenous and relevant conditions. Initial experiments suggest that ACX3 cannot function in place of IBR3. ACX3 expression patterns are likely too different from IBR3 to have overlapping function. This can be interpreted as evidence for an indirect role of ACX3 in IBA metabolism.

If ACX3 and IBR3 together oxidize IBA-CoA, I could expect enhancement to be limited to this combination of mutants. However, acx3 enhances the IBA resistant phenotypes of other ibrs and ibr3 enhances the IBA resistant phenotypes other acx mutants (Figure 5-7). These results would support an indirect role of ACX3 in IBA metabolism. However, the IBA phenotype of ibr3 is enhanced when combined with other
ibrs. Genetically, this suggests an alternate pathway and that IBR3 may not act alone to oxidize IBA-CoA.

Although I did not conclusively determine the mechanism of ACX3’s influence in IBA metabolism, I have generated genetic tools that will provide complementary evidence when paired with biochemical data. Pilot experiments of promoter swapping of ACX3 and IBR3, varying phenotypes of acx ibr3 mutants, and reduced acyl-CoA activity of ibr3 acx3 suggest that ACX3 likely influences IBA metabolism indirectly. Direct testing of ACX3 activity on IBA-CoA substrate will be the most direct way to determine if ACX3 directly or indirectly affects IBA metabolism. in vitro assays of ACX3 activity on IBA are now possible after the identification of LACS4 which was unfortunately identified and assayed years after my work on ACX3 halted.
6. Discussion

The limited mobility of plants makes their propensity for adaptive plasticity to respond to changes in their local environment essential to their survival. Root systems are adaptive and adopt particular growth patterns in response to environmental cues. For instance, roots in phosphate-starved conditions develop more elongated lateral roots near the soil surface and elongated root hairs in efforts to uptake more phosphate (Williamson et al. 2001). Plants accomplish this modulation of root growth through an integral network of hormone signaling pathways (Gray 2004; Chapman and Estelle 2009; Dastidar et al. 2012; Bhosale et al. 2018).

The auxin class of hormones that controls cell elongation and division, making them essential to plant growth and response to environmental cues (Mroue et al. 2018). IBA, a storage form of auxin, can be converted to IAA, the primary signaling auxin. IAA derived from IBA is the main source of auxin that tells the plant to develop lateral roots, indicating that this pathway is fundamental to root morphology (De Rybel et al. 2012; Xuan et al. 2015). IBA may also contribute to root plasticity in changing environmental conditions by contributing to root hair elongation in response to phosphate starvation (Trujillo-Hernandez et al. 2020).

IBA is metabolized to IAA via a stepwise mechanism similar to fatty acid β-oxidation in the peroxisome. Proper utilization of IBA requires functional enzymes to directly convert IBA into IAA and functional peroxisomes. Peroxisomes have function in a diverse array of processes, making them important throughout the life span of a plant because they are necessary for fatty acid β-oxidation, photorespiration, and synthesis of the hormones IBA, JA, and SA (Hu et al. 2012; Kao et al. 2018).
Levels of IBA appear to be regulated since IBA exists in conjugated forms and there are carrier proteins that transport IBA out of the plant and sequester it in the vacuole (Damodaran and Strader 2019; Michniewicz et al. 2019). These are potential mechanisms to limit the amount of IBA available for β-oxidation ultimately influencing root architecture. When IBA is available to the plant, it is metabolized into IAA in a stepwise process in the peroxisome. It is through this β-oxidation process that IBA shapes root architecture. Disruptions to IBA homeostasis result in altered root morphology. Mutations to IBA transporters and IBA conjugation result in plants hypersensitive to IBA with increased lateral root density, while disruptions to IBA β-oxidation result in resistance to IBA and reduced lateral root density.

Although many genes necessary for proper control of IBA levels and IBA metabolism have been identified, much remains unknown. I conducted an enhancer screen in an effort to identify novel genes in IBA metabolism. From this screen, I pulled seven enhancer mutants from the ibr3-1 background and five from the ibr1-1 background.

To identify the causative mutations, I combined the traditional mapping technique with the more modern whole genome sequencing approach. Several enhancer mutants were outcrossed to WS to generate mapping populations to identify the relative genomic region of the causative mutation. Rough mapping could be useful when paired with whole genome sequencing to narrow down the candidate genes in the sequencing data. To see linkage in a mapping population, the marker tested needs to be relatively close to the causative mutation. Rough mapping, utilizing approximately three markers across the chromosomes, did not provide enough resolution to pinpoint the location of the causative
mutations. My positive identification of two enhancer mutants was accomplished using sequencing data alone. To generate genomic diversity in noncausative SNPs, the enhancer mutants were backcrossed to wild type. Pooled DNA from independently isolated F3 enhancers from the backcross to wild type was sufficient to segregate noncausative mutations, decrease the number of noncausative homozygous mutations, and reduce the number of candidate genes while maintaining confidence in the sequenced lines.

I successfully identified LACS4 and ACX3 from the sequencing data of two enhancer mutants, while 10 mutants from my enhancer screen remain unidentified. Sequencing data from the unidentified mutants did not reveal obvious candidate genes, suggesting that they are defective in novel genes to IBA metabolism. Successful identification of these mutants will greatly expand our knowledge of IBA metabolism and peroxisome function.

6.1. LACS4

Identification of LACS4 completes the enzymatic components of IBA β-oxidation and opens the field of IBA metabolism to study the enzymatic reactions of IBA metabolism in detail. The synthetase that charges IBA with CoA was previously been unidentified. While characterizing LACS4, I discovered that LACS6 may also function in concert with LACS4 to charge IBA with CoA. lacs4 is resistant to IBA in primary root and hypocotyl elongation, but remains sensitive to IBA stimulation of lateral root initiation. Recombinant LACS4 had activity on IBA, but not IAA, tryptophan, IAA conjugates, or 2-4-DB, demonstrating specific activity for IBA as a substrate. DR5:GUS induction was reduced in lacs4 and lacs4 ibr3 when treated with IBA, which supports my
hypothesis that LACS4 contributes to the root cap auxin pool through its actions on IBA, demonstrating it has a physiologically relevant effect on plant development.

Evidence that \textit{lacs4} is resistant to IBA in primary root elongation and has activity on IBA \textit{in vitro} strongly support the hypothesis that LACS4 directly functions to metabolize IBA. Our working model of IBA metabolism now includes LACS4 and LACS6 as the CoA oxidases that function in IBA metabolism (Figure 6-1). This work expands the function of LACS4 to span lipid and hormone metabolism. The primary function of LACS4 may be in IBA metabolism since \textit{lacs4} single mutants have phenotypes on IBA, while defects in lipid synthesis are only detected in \textit{lacs4} double mutants. This work expands the breadth of LACS4 function in plants to now include including root development, vegetative growth, seed oil accumulation, fertility, and IBA \(\beta\)-oxidation.

6.2. ACX3

\textit{in vitro} evidence that LACS4 directly accepts IBA as a substrate is directly contrary to ACX3, the other mutant identified in the enhancer screen. It has been suggested that ACX3 indirectly influences IBA metabolism (Adham et al. 2005; Rafeiza et al. 2012) since ACXs have preference for fatty acids of distinct lengths, although definitive evidence has not been presented. \textit{acx3} strongly enhances \textit{ibr3} resistance to IBA and has been identified twice in enhancer screens. ACX3 clearly influences IBA metabolism but the mechanism is unclear. I attempted to answer if ACX3 directly or indirectly functions in IBA metabolism utilizing genetic approaches.

One hypothesis is that IBA can fit into the binding pocket of ACXs and is accepted as a substrate. The alternative hypothesis is that \textit{acxs} disrupt IBA metabolism
indirectly through CoA limitations or accumulation of byproducts that slows metabolism in general. *acx3* enhances resistance to IBA when combined with mutations to other *ibr*s, while enhancement is variable when *ibr3* is combined with other *acxs*. This supports previous knowledge that the ACX family have variable contributions to IBA metabolism (Adham et al. 2005; Rafeiza et al. 2012). ACX3 and ACX1 are more highly expressed in imbibed seeds and early in seedling development than ACX4 and ACX5 may explain why *acx1* and *acx3* have the strongest IBA resistant phenotypes on their own and enhance *ibr3*. Disruptions to internal peroxisome content early in development may compromise peroxisome metabolism thereafter.

Alternatively, ACX1 and ACX3 may be able to accept IBA-CoA as a substrate. If ACXs can accept the same substrate as IBR3, IBR3 may be able to accept the same substrates as ACXs. I did not find phenotypic evidence that IBR3 oxidizes fatty acids, as *ibr3* and *ibr3* in combination with any *acx* are not sucrose dependent. However, extracts isolated from *ibr3* *acx3* have reduced acyl-CoA oxidase activity on C12:0 substrates. This could indicate that *ibr3* does disrupt fatty acid β-oxidation but can only be detected at a biochemical level, or that internal peroxisome dynamics are compromised in *ibr3* *acx3*, resulting in reduced or slowed metabolism. This evidence alone could support either hypothesis depending on interpretation.

*ACX3* expressed in planta under control of *IBR3* promoter failed to rescue the IBA resistant phenotypes of *ibr3* and *acx3*, suggesting ACX3 cannot function in place of IBR3 and the expression pattern is likely too different than *IBR3* to function in concert with IBR3 as an IBA acyl-CoA oxidase in vivo. Although *ACX3* failed to complement
*ibr3 in planta*, this does not conclusively demonstrate that ACX3 does not oxidize IBA-CoA.

More direct attempts to investigate the alternative hypothesis that *acx3* disrupts IBA metabolism indirectly, perhaps by CoA limitations, were conducted by overexpressing what was thought to be the CoA transporter and an acyl-CoA thioesterase. Shortly after the *PXN* overexpression constructs were generated, investigations by other groups revealed that PXN does not transport CoA under physiologically relevant conditions (van Roermund et al. 2016). Overexpression of *ACH2* did not rescue the IBA resistant phenotype of *acx3*. Measurements of peroxisomal CoA levels is necessary to determine if 35S:*ACH2* and 35S:*PXA1* increased CoA levels and determine if this experimental design was successful.

Together, the data from genetically manipulating ACX3 is more supportive of an indirect role of ACX3 in IBA metabolism, although it does not convincingly support or refute either hypothesis for the role of ACX3 in IBA metabolism on their own. The model has been updated to remove ACXs as oxidases in IBA metabolism (Figure 6-1). Data from these lines will be useful in combination with techniques to measure contents of the peroxisome and *in vitro* enzymatic analysis to conclusively determine how ACX3 influences IBA metabolism.
Figure 6-1. Updated model of peroxisome β-oxidation pathways.

LACS4 and LACS6 are now listed as the CoA synthetases that function in IBA metabolism. LACS6 functions in both IBA metabolism and fatty acid β-oxidation. ACXs are now thought to function in fatty acid β-oxidation and JA synthesis but not IBA metabolism.
6.3. Implications for future screens for IBA resistance

The original screen for plants resistant to the effects of IBA was published 20 years ago (Zolman et al. 2000) and led to the identification of numerous genes specific to IBA metabolism, general peroxisome function, and enzymes with characterized function in other peroxisomal pathways. Continuation of the screen has led to the identification of multiple alleles of genes already known to be resistant to IBA. For instance, four mutant alleles of *IBR3* have been identified in forward genetic screens (Zolman et al. 2007). However, the screen is not yet saturated, implying that there are more genes to discover in IBA metabolism.

To increase the chance of discovering genes with novel roles in IBA metabolism, an enhancer screen was conducted. I was successful and identified LACS4 which was a novel discovery to IBA metabolism as the CoA synthetase functioning in IBA metabolism was previously unknown. LACS4 was not identified as a candidate to test using reverse genetics because it has no known association with the peroxisome. All known genes identified with roles in IBA metabolism are associated with the peroxisome, creating an inherent bias against genes with no known peroxisomal function, such as LACS4.

The enhancer screen demonstrates that forward genetics is still a useful technique to identify new players in IBA metabolism. Several new enhancer mutants were pulled from the screen for enhanced resistance to IBA. Whole genome sequencing data of several of these mutants did not uncover clear candidate mutations. Two of these mutants, HRI#11 *ibr1-1* and HRI#41 *ibr1-1*, remained unidentified even though I possess the data of every gene mutated in these mutants. The fact that HRI#11 *ibr1-1* does not
have mutations in genes with known function in hormone metabolism, peroxisome function, or even obvious candidates tells us that the causative mutation may be defective in a novel gene and supports the hypothesis that there are many more players important for IBA metabolism to discover. Forward genetics will continue to be a useful and successful tool to identify new genes in IBA metabolism.

Further genetic analysis of mutants allows better understanding how components of the peroxisome, peroxisome pathways, and IBA metabolism work together. ECH2 had been identified in a forward genetic screen for IBA resistance and was thought to be an enoyl-CoA hydratase that functions in IBA metabolism and fatty acid β-oxidation (Strader et al. 2011), but diligent experiments uncovered that the mechanism of IBA resistance of ech2 is more complex. The ech2 mfp2 double mutant surprisingly suppressed the IBA phenotype of ech2 and additional experiments support that the IBA resistance of ech2 is due to an indirect effect of accumulation of fatty acid intermediates (Li et al. 2019). The detailed study of ech2 provides better insight to how the dynamics of peroxisomes metabolic pathways can influence each other and would only have been teased apart using genetics.

Forward genetics will remain crucial technique to discover novel genes in IBA metabolism and help characterize peroxisome function. Although I failed to identify all the enhancer mutants I pulled from the enhancer screen through sequencing alone, I was able to conclude that the causative mutations in these enhancers could be defective in a gene novel to IBA metabolism and peroxisome function. There is much more knowledge to be gained about IBA metabolism and peroxisome function.
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